APPENDIX A

VERIFICATION OF TRANSLATION

I, Sophie Deléhouzée

of 6th Fl., Kantetsu Tsukuba-Science-City Bldg. 1-1-1, Oroshi-machi, Tsuchiura, Ibaraki, JAPAN

declare as follows:

1. That I am well acquainted with both the English and Japanese languages, and

2. That the attached document is a true and correct translation of a certified copy of the following application, which was made by me to the best of my knowledge and belief of.

Japanese Patent Application No. 2004-010971
Entitled: "INFLAMMATORY CYTOKINE INHIBITORS"
Filed on January 19, 2004

January 5, 2009
Date

Sophie Deléhouzée
Sophie Deléhouzée

【Name of Document】 APPLICATION FOR PATENT
【Identification Number】 M3-A0305
【Filing Date】 January 19, 2004
【Addressee】 Esq. Commissioner of the Patent Office
【Inventor】
【Address】 407-3, Obara, Takatomachi, Kamiina-gun, NAGANO
【Name】 OKABE, Ayako
【Inventor】
【Address】 5861-3, Tomigata, Ina-shi, NAGANO
【Name】 TOJI, Shingo
【Inventor】
【Address】 304, Space Arc Hill II, 3813-1, Nishiminowa, Ina-shi, NAGANO
【Name】 KISHI, Yoshiro
【Inventor】
【Address】 1-5-22, Fujizuka, Kohoku-ku, Yokohama-shi, KANAGAWA
【Name】 YAHARA, Ichiro
【Applicant】
【Identification Number】 390004097
【Name or Appellation】 MEDICAL AND BIOLOGICAL LABORATORIES CO., LTD.
【Attorney】
【Identification Number】 100102978
【Patent Attorney】
【Name or Appellation】 Hatsushi Shimizu
【Nominated Attorney】
【Identification Number】 100108774
【Patent Attorney】
【Name or Appellation】 Kazunori Hashimoto

【Payment】
【Registration Number】 041092
【Amount】 21000 yen
【List of Attached Documents】
【Name of Document】 Claims 1
【Name of Document】 Specification 1
【Name of Document】 Drawings 1
【Name of Document】 Abstract 1

[Document Name] Claims

[Claim 1]

A substance, or a derivative thereof, having an ability to bind to a CD61 protein and an inhibitory effect on inflammatory cytokine production.

[Claim 2]

The substance or derivative of claim 1, wherein the substance is a protein.

[Claim 3]

The substance or derivative of claim 1, wherein the substance is an antibody.

[Claim 4]

The substance or derivative of claim 1, 2, or 3, wherein the inflammatory cytokine is any one of IFN-γ, TNFα, IL-1, and IL-6.

[Claim 5]

The substance or derivative of claim 1, 2, 3, or 4, having an IL-10 production-inducing effect.

[Claim 6]

An inhibitor of inflammatory cytokine production comprising as an effective ingredient the substance or derivative of any one of claims 1 to 5.

[Claim 7]

A pharmaceutical for preventing or treating an inflammatory disease, wherein the pharmaceutical comprises the inhibitor of inflammatory cytokine production of claim 6.

[Claim 8]

A pharmaceutical for preventing or treating hypercytokinemia, wherein the pharmaceutical comprises the inhibitor of inflammatory cytokine production of claim 6.

[Claim 9]

A method for inhibiting inflammatory cytokine production using the substance or derivative of any one of claims 1 to 5.

[Claim 10]

A method for judging the effectiveness of the pharmaceutical of claim 7 or 8 in treating an inflammatory disease or hypercytokinemia, wherein the method comprises the step of contacting a test sample with an anti-CD61 antibody.

[Claim 11]

A kit for judging the effectiveness of the pharmaceutical of claim 7 or 8 in treating an inflammatory disease or hypercytokinemia.

[Claim 12]

A method of screening for a substance having an ability to bind to a CD61 protein and an inhibitory effect on inflammatory cytokine production, wherein the method comprises the

steps of:

(a) contacting an inducer of cytokine production and a test substance with a CD61-expressing cell; and

(b) measuring the inflammatory cytokine level, comparing it with that of a control contacted with only the inducer of cytokine production, and selecting a test substance that reduced the cytokine level produced.

[Document Name] Specification

[Title of the Invention] INFLAMMATORY CYTOKINE INHIBITORS

[Technical Field]

[0001]

The present invention relates to inflammatory cytokine inhibitors using an anti-CD61 antibody.

[Background Art]

[0002]

Inflammation is a complex series of reactions expressed by living organisms in response to stimuli. Causes of inflammation are varied: for example, exogenous factors including pathogenic microorganisms such as bacteria and viruses, physical factors such as external injury and radiation, and chemical factors such as acids and alkalis trigger inflammation. Inflammation is essentially one of the defense reactions of organisms. It can also be induced by endogenous causes: for example, immune complexes generated by endogenous autoantigens cause autoimmune diseases.

[0003]

A large number of diseases accompany inflammatory symptoms, one example of which is sepsis. Sepsis is a disease showing a systemic inflammatory reaction in response to infection, often triggered by infection involving burns, external injury, highly invasive surgery, and diseases such as cancer and pneumonia. During sepsis, an abnormal increase in inflammatory cytokine concentration in blood is observed (hypercytokinemia). In serious cases, a self-destructive systemic inflammation occurs, thrombi are formed and multiple organs become dysfunctional in a short period of time. Death results in worst cases.

[0004]

In spite of being such a serious disease, few effective treatment methods are available for sepsis. Fatal sepsis cases are numerous, and every year, 210,000 people die of sepsis in the United States. Sepsis is ranked as the number one cause of death in the intensive care unit apart from cardiac diseases. Only limited treatment methods are

clinically applied at present, examples being endotoxin absorption-mediated blood purification methods, and administration of, as a pharmaceutical, activated protein C (Xigris™, Eli Lilly and Company) having anticoagulant and anti-inflammatory effects.

[0005]

Inflammatory cytokines are cytokines that promote inflammation. "Cytokine" is a general term for a variety of physically active substances responsible for intercellular signal transductions. Cytokines form a group of substances involved in regulating various biological functions by participating in intracellular signal transduction via specific receptors.

[0006]

Examples of biological functions in which cytokines are involved include biological defense and immune responses (Non-Patent Document 1). Of these, inflammation which is primarily an organism's defense reaction is deeply associated with cytokines. Among cytokines, TNFα, IL-6, IL-1, IL-12, and the like act to promote inflammation, and therefore, are classified as inflammatory cytokines. In addition, cytokines having an activity to regulate the production of these inflammatory cytokines are referred to as anti-inflammatory cytokines (Non-Patent Documents 2 and 3). These inflammatory cytokines and anti-inflammatory cytokines are known to appropriately regulate their mutual production level and activities (Non-Patent Document 3).

[0007]

IL-10 is an anti-inflammatory cytokine. Since IL-10 is expected to exert inhibitory effects on the production of the inflammatory cytokines TNFα and IFN-γ, clinical trials of IL-10 administration to psoriasis and Crohn's disease patients are being carried out, and some positive results have been obtained (Non-Patent Documents 8 and 9). However, in contrast to these findings, it has also been reported that administration of a large dose of exogenous IL-10 has the risk of aggravating inflammation (Non-Patent Document 10). Therefore, the treatment of inflammatory diseases aiming at the inhibitory effect of IL-10 on inflammatory cytokines is not easy.

[Non-Patent Document 1] Atsushi Miyajima, Toshio Kitamura and Naoko Arai, Molecular Biology of Cytokines, Yodosha 1995

[Non-Patent Document 2] Moore, K.W., et al., Annu. Rev. Immunol. 19:683-765, 2001

[Non-Patent Document 3] Mosmann, T.R. Adv. Immunol., 56:1-26, 1994

[Non-Patent Document 4] Shigeo Koyasu, Menekigaku ga wakaru (All about Immunology), pp74-80, Yodosha, 2000

[Non-Patent Document 5] Mosmann, T.R. Ann. NY Acad. Sci.,664:89-92, 1992

[Non-Patent Document 6] Bloom, B.R et al., Ann. Rev. Immunol., 10:453-488, 1997

[Non-Patent Document 7] Klingemann, H.G. and Dedhar, S., Blood 74(4):1348-1354, 1989
[Non-Patent Document 8] Kimball, A.B., et al., Arch Dermatol 138(10):1341-1346, 2002
[Non-Patent Document 9] Van Deventer, S.J.H., et al., Gastroenterology, 113: 383-389, 1997
[Non-Patent Document 10] Lauw, F.N., et al., The Journal of Immunology 2000, 165:2783-789

[Disclosure of the Invention]
[Problems to be Solved by the Invention]

[0008]

The present invention was achieved in view of the above circumstances. An objective of the present invention is to provide a novel therapeutic drug for inflammatory diseases including sepsis.

[Means for Solving the Problems]

[0009]

To solve the above-described problems, the present inventors conducted a dedicated search for candidate substances of inflammatory disease therapeutic drugs. The present inventors, who focused on the relationship between inflammatory diseases and cytokines, came up with the idea of obtaining functional antibodies that would endogenously regulate cytokines. Mice were immunized with a human peripheral blood monocyte fraction (PBMC), and cytokine-regulating effects of the obtained antibodies were examined. As a result, it was confirmed that, of these antibodies, antibody #33 significantly inhibited IFN-γ production. At the same time, the present inventors confirmed the IL-10 production-promoting effect of antibody #33.

[0010]

As described above, the present inventors further examined the IL-10 production-promoting effect of antibody #33 since results contrary to expectations are being reported for exogenous IL-10 administration. As a result, antibody #33 was found to have the effect of accelerating IL-10 production, but no activity to induce excessive IL-10 production.

[0011]

Furthermore, when antibody #33 was further examined for regulatory effects on other cytokines, it was found to inhibit the production of many typical inflammatory cytokines.

[0012]

These results showed that, in addition to having no risk of inducing inflammation due to excessive IL-10 production, antibody #33 of the present inventors definitely inhibits

the production of typical inflammatory cytokines. If antibody #33 is applied to the treatment of inflammatory diseases, it may become a pharmaceutical having both a definite efficacy and a high safety. Furthermore, antigen investigations for this promising antibody revealed that the antigen is CD61. In other words, the present inventors discovered that a substance binding to CD61 possesses an inhibitory effect on inflammatory cytokine production and generated a novel therapeutic drug for inflammatory diseases using this substance. Specifically, the present invention provides the following:

(1) a substance, or a derivative thereof, having an ability to bind to a CD61 protein and an inhibitory effect on inflammatory cytokine production;

(2) the substance or derivative of (1), wherein the substance is a protein;

(3) the substance or derivative of (1), wherein the substance is an antibody;

(4) the substance or derivative of (1), (2), or (3), wherein the inflammatory cytokine is any one of IFN-γ, TNFα, IL-1, and IL-6;

(5) the substance or derivative of (1), (2), (3), or (4), having an IL-10 production-inducing effect;

(6) an inhibitor of inflammatory cytokine production comprising as an effective ingredient the substance or derivative of any one of (1) to (5);

(7) a pharmaceutical for preventing or treating an inflammatory disease, wherein the pharmaceutical comprises the inhibitor of inflammatory cytokine production of (6);

(8) a pharmaceutical for preventing or treating hypercytokinemia, wherein the pharmaceutical comprises the inhibitor of inflammatory cytokine production of (6);

(9) a method for inhibiting inflammatory cytokine production using the substance or derivative of any one of (1) to (5);

(10) a method for judging the effectiveness of the pharmaceutical of (7) or (8) in treating an inflammatory disease or hypercytokinemia, wherein the method comprises the step of contacting a test sample with an anti-CD61 antibody;

(11) a kit for judging the effectiveness of the pharmaceutical of (7) or (8) in treating an inflammatory disease or hypercytokinemia; and

(12) a method of screening for a substance having an ability to bind to a CD61 protein and an inhibitory effect on inflammatory cytokine production, wherein the method comprises the steps of:

(a) contacting an inducer of cytokine production and a test substance with a CD61-expressing cell; and

(b) measuring the inflammatory cytokine level, comparing it with that of a control contacted with only the inducer of cytokine production, and selecting a test substance that reduced the cytokine level produced.

[Effects of the Invention]

[0013]

The present invention has provided novel inflammatory cytokine inhibitors. Substances provided by the present invention can be used as useful pharmaceuticals in preventing or treating inflammatory diseases and hypercytokinemia via an inhibitory effect on inflammatory cytokines.

[Best Mode for Carrying Out the Invention]

[0014]

The present invention provides substances (hereinafter referred to as "substances of the present invention"), or derivatives thereof, having a CD61 protein binding ability and an inhibitory effect on the production of inflammatory cytokines. The present invention is based on the discovery by the present inventors that an anti-CD61 antibody, or substances having a CD61 protein binding ability, inhibit the production of various inflammatory cytokines.

[0015]

In general, "inflammatory cytokine" is a general term for cytokines causing inflammatory symptoms, good examples of which being IL-1, IL-6, IFN-γ, and TNF-α. In the present invention, the inhibitory effect on the production of inflammatory cytokines is not limited to the above-described examples and any one of the inflammatory cytokines can be inhibited. The substances may also have an inhibitory effect on the production of a plurality of inflammatory cytokines, either simultaneously or around the same time.

[0016]

CD61 is a protein known as a cell surface molecule subunit and is also called integrin β_3-subunit and integrin β_3 chain. CD61 forms complexes with CD41 and CD51, i.e. CD41/CD61 and CD51/CD61, which function as cell surface molecules belonging to the integrin family. CD41/CD61 is also referred to as αIIbβ3, which is expressed specifically on platelets and megakaryocytes, functions in platelets as a receptor for fibrinogen and von Willebrand factors, and is a core factor in platelet aggregation and platelet adhesion. In addition, CD51/CD61 is also referred to as αVβ3 and is present on vascular endothelial cells, osteoclasts, macrophages, platelets, etc. These molecules are involved in cell adhesion, proliferation, and migration in the vascular endothelium and such, and also in angiogenesis, intimal hypertrophy of the blood vessels, etc. The binding region(s) and binding intensity do not matter when it comes to the CD61 protein binding ability according to the present invention, as long as an inflammatory cytokine inhibitory effect exists.

[0017]

Since the substances of the present invention have an inhibitory effect on the production of inflammatory cytokines, administration of the substances may be effective in treating or preventing hypercytokinemia. Hypercytokinemia refers to a pathological condition in which cytokines in the blood have increased, and is observed in diseases such as sepsis, systemic inflammatory response syndrome (SIRS), hemophagocytosis syndrome (HPS), and Kawasaki disease, as well as in cases of surgical invasion. Furthermore, the substances of the present invention may be applied to the treatment or prevention of inflammatory diseases including sepsis. As described above, inflammatory cytokines are known to be involved in the establishment of inflammatory diseases, the aggravation of sepsis, etc. Inflammatory diseases refer to diseases showing inflammatory symptoms and are exemplified by sepsis and autoimmune diseases. Furthermore, systemic inflammatory response syndrome (SIRS), sepsis caused by infection, septic shock, and severe sepsis are all comprised in "sepsis" of the present invention.

[0018]

Examples of such substances of the present invention include deoxyribonucleic acids, ribonucleic acids, proteins, peptides, and low-molecular substances. For example, fibrinogen which is a ligand for CD61, the RGD sequence which is its epitope, and derivatives thereof have a potential to become a substance of the present invention. As a preferred example, an anti-CD61 antibody may be given.

[0019]

The anti-CD61 antibodies of the present invention may be either polyclonal or monoclonal antibodies; however, they are preferably antibodies that are uniformly and stably produced. In addition, chimeric antibodies, humanized antibodies, human antibodies, antibody fragments, and mutants of the anti-CD61 antibodies are also examples of the anti-CD61 antibodies that can be used in the present invention. An antibody fragment refers to a portion of a full-length antibody and generally refers to a fragment comprising an antigen-binding region or a variable region. Fab and $F(ab')_2$ are preferable examples of antibody fragments of the present invention.

[0020]

As long as the anti-CD61 antibodies of the present invention have an inhibitory effect on the production of inflammatory cytokines, it does not matter what epitope the antibody binds. Examples of such anti-CD61 antibodies include clone SZ21 and antibody #33. An example of a preferred antibody is antibody #33.

[0021]

Antibody #33 of the present invention is an anti-CD61 antibody, for which the antigen is CD61. Of the anti-PBMC antibodies prepared by the present inventors, antibody

#33's inhibitory effects on various inflammatory cytokines were specifically confirmed herein. Regarding antibody #33, the isotype of the antibody #33 is IgG2a, κ. When developed by SDS-PAGE (containing a reducing agent), bands can be observed at positions of approximately 55 kDa for the H chain and approximately 30 kDa for the L chain. Antibody #33 has been confirmed to work in an inhibitory manner towards at least IFN-γ, TNFα, IL-6, IL-1α, and IL-1β, as described in detail in the Examples. Furthermore, antibody #33 has an IL-10 production-inducing effect in the present invention as described below.

[0022]

The anti-CD61 antibodies can be prepared by known methods using CD61 or PBMC as antigen. In the case of polyclonal antibodies, antibodies can be produced by injecting animals such as rabbits and rats with the antigen and the collected blood can be purified by affinity chromatography. In the case of monoclonal antibodies, the hybridoma method (Kohler and Milstein, Nature 256: 495(1975)), the recombinant method (U.S. Patent No. 4816567), or such may be used. Moreover, anti-CD61 antibody mutants can be prepared by random mutagenesis, in which random mutations are introduced into specific genetic DNAs by the PCR method and such to produce mutants, or by the chain shuffling method, which screens for combinations of variable regions of antibodies with a high specificity to the original antigen, and in which the antibody variable region of either the VH gene or the VL gene is fixed and the other is linked to a V gene library to construct and express a library. $F(ab')_2$ are prepared by digesting IgG with pepsin and removing the Fc portions. In this case, the pH, the duration of digestion, and the amount of pepsin are appropriately adjusted.

[0023]

Whether the prepared antibody is an anti-CD61 antibody can be confirmed by examining the affinity with the CD61 antigen. Confirmation of the affinity with the antigen is usually performed as a screening of the antibody at the time of preparation of the antibody. The affinity of an antibody can be determined by saturation binding, enzyme-linked immunosorbent assay (ELISA), or such.

[0024]

Other substances of the present invention can be prepared by known methods in addition to the screening method described below. For example, when the substance of the present invention is a protein, proteins that bind to the CD61 protein can be screened from a group of candidate substances by known methods such as the West-western method, the two-hybrid method, the immunoprecipitation method, or such. Screening can also be performed using an apparatus (for example BIACORE) for analyzing the molecular

interactions by surface plasmon resonance (SPR). Substances screened in this way can be further selected after confirming their inhibitory effect on the production of inflammatory cytokines and purified using affinity columns and such to prepare a substance of the present invention. The verification of the inhibitory effect on the production of inflammatory cytokines can be done as described in the Examples, by contacting a subject substance with inflammatory cytokine-producing cells, comparing the production level of the inflammatory cytokines by these cells before and after the contact with the subject substance, and examining whether the production level after the contact has decreased compared to before the contact. The level of inflammatory cytokines can be measured by various immunoassays that use anti-cytokine antibodies, such as the HPLC method and the ELISA method.

[0025]

Furthermore, when the substance of the present invention to be prepared is a small molecule, it can be prepared, for example, by subjecting the small molecules of a library constructed by combinatorial chemistry to a high throughput screening by known methods (Wrighton NC; Farrell FX; Chang R; Kashyap AK; Barbone FP; Mulcahy LS; Johnson DL; Barrett RW; Jolliffe LK; Dower WJ.; Small peptides as potent mimetics of the protein hormone erythropoietin, Science (UNITED STATES) Jul 26 1996, 273 p458-64; Verdine GL., The combinatorial chemistry of nature, Nature (ENGLAND) Nov 7 1996, 384 p11-13; Hogan JC Jr., Directed combinatorial chemistry, Nature (ENGLAND) Nov 7 1996,384 p17-9).

[0026]

The substances of the present invention may also have an IL-10 production-inducing effect. The IL-10 production-inducing effect of the present invention comprises the effect of accelerating the initiation time point of IL-10 production and secretion from IL-10 producing cells. When the effect of accelerating the initiation time point of IL-10 production and secretion is confirmed, the substance is judged to have the IL-10 production-inducing effect of the present invention even if the total amount of IL-10 production at the time of addition or administration of the substance did not increase compared to when no substance was added or administered. Confirmation of the IL-10 production-inducing effect can be done, as described in the Examples, by measuring the amount of IL-10 secreted from IL-10 producing cells by various known immunoassays such as the ELISA method using an anti-IL-10 antibody. Furthermore, the presence of the effect of accelerating the initiation time point of IL-10 production and secretion can be verified by monitoring over a period of time.

[0027]

The present invention also provides derivatives of the above-described substances of the present invention. Examples of derivatives of the substances of the present invention include prodrugs of the substances of the present invention. Such prodrugs are substances for which the derivative itself has no CD61 protein-binding ability and/or no inflammatory cytokine production-inhibitory effect, but which become substances exhibiting a CD61 protein-binding ability and an inflammatory cytokine production-inhibitory effect after administration into the body. The prodrug derivatives of the above-described substances are considered to be useful in treating or preventing inflammatory diseases and hypercytokinemia, similarly to the above-described substances.

[0028]

When the substances of the present invention, or derivatives thereof, are used as pharmaceuticals, they can be appropriately formulated according to standard methods. These formulations may comprise pharmacologically acceptable carriers and additives. For example, surfactants, excipients, stabilizers, preservatives, suspending agents, isotonizing agents may be comprised. Considering the administration route and the patient, a suitable dosage form can be selected among known dosage forms such as injections, tablets, capsules, microcapsules, etc.

[0029]

The present invention provides methods for inhibiting the production of inflammatory cytokines using a substance of the present invention or a derivative thereof. The methods of the present invention can be performed *in vivo* or *in vitro*. For example, a substance of the present invention or a derivative thereof can be administered to experimental animals or cultured cells to inhibit the production of inflammatory cytokines in these experimental animals or cultured cells. The methods of the present invention may thereby become methods for elucidating biological reactions in which cytokines are involved. Furthermore, as described above, these methods may become effective methods, not only in the field of research, but also in the field of medicine. In other words, by administering a substance of the present invention, or a derivative thereof, to patients with inflammation or hypercytokinemia or patients with such predispositions, the substance or derivative can be used to prevent and treat inflammatory diseases and hypercytokinemia.

[0030]

Moreover, the present invention provides a method for judging the efficacy of the above-described pharmaceuticals in treating inflammatory diseases or hypercytokinemia. The method of judgment of the present invention enables one to judge whether the treatment with the above-described pharmaceutical is effective or not for each patient prior to *in vivo* treatment, and can provide the clinical scene with so called tailor-made treatment.

Thrombasthenia can be given as an example of a disorder whose association with a CD61 mutation has been clarified. Due to a type of CD61 mutation affecting fibrinogen-binding, platelet aggregation is inhibited, thus causing a tendency to bleed. The above-described CD61 mutation is known to have a single amino acid sequence mutation. Types D119Y (amino acid 119 is mutated from D to Y; same is true for the other types), R214Q, R214W, and S752P have so far been identified. In addition, L33P, R143Q, P407A, R439Q, and R636C are known as asymptomatic mutations. If disease onset is related to inflammatory cytokines, it is conceivable that the methods of the present invention can likely be applied.

[0031]

The methods of the present invention comprise a step of contacting a test sample with an anti-CD61 antibody.

An example of a method of the present invention is as follows: first, an inducer of cytokine production is added to peripheral blood or peripheral blood monocytes collected from a subject (hereinafter referred to as subject's sample) to induce inflammatory cytokine production. As an inducer of cytokine production, for example, LPS can be used. Next, the subject's sample treated as described above is divided, and an anti-CD61 antibody is added to one part while the other is used as a control. The cytokine production level in both samples is measured by ELISA or such. The cytokine to be measured can be selected from inflammatory cytokines. When the inflammatory cytokine level in the sample to which the anti-CD61 antibody has been added is lower than that in the control, the treatment with the above-described pharmaceutical is judged to have been effective.

[0032]

As a different example, the level of cytokine production in a subject's sample is measured, then an anti-CD61 antibody is added to this sample, and then the level of cytokine production in the sample after antibody addition is measured by ELISA, or such. The measured values before and after antibody addition are compared, and when the value after antibody addition is lower than that before antibody addition, the treatment with the above-described pharmaceutical is judged to have been effective. This method can be applied to, for example, patients in whom an increase of the level of cytokines in blood has already been confirmed.

[0033]

Although peripheral blood or peripheral blood monocytes has been cited as a preferred example of a subject's sample, the test sample is not limited thereto, and affected tissues in which an inflammation is occurring and such may be used.

[0034]

Reagents necessary for a method of the present invention may be combined in advance as a kit. One of the reagents constituting the kit comprises an anti-CD61 antibody as an ingredient. Besides this, the kit may comprise a reagent for measuring a cytokine, a cytokine inducer, an appropriate diluent, etc, as required. Although they may vary according to the method for measuring cytokines, for example in the case of the ELISA method, the reagents for measuring cytokines are exemplified by reagents comprising as an ingredient an anti-cytokine antibody, an enzyme-labeled antibody, or a substrate.

[0035]

The present invention provides methods of screening for the above-described substances of the present invention. A screening method of the present invention comprises the steps of: contacting CD61-expressing cells with an inducer of cytokine production and a test substance; and measuring the inflammatory cytokine level, comparing with a control contacted only with the inducer of cytokine production, and selecting a test substance that reduced the produced cytokine level.

[0036]

"The step of contacting CD61-expressing cells with an inducer of cytokine production and a test substance" is explained: For example, CD61-expressing cells are seeded into a 96-well plate and an inducer of cytokine production and a test substance are added to each well simultaneously or in tandem to contact these substances with CD61-expressing cells. Herein, a test substance is a substance that is the object of the screening. Examples of CD61-expressing cells include PBMC, and examples of an inducing substance of cytokine production include LPS. In addition, CD61-expressing cells contacted with only the substance inducing cytokine production, without contacting the test substance, are prepared as a control. Next, the "step of measuring the inflammatory cytokine level, comparing with a control contacted only with the inducer of cytokine production, and selecting a test substance that reduced the produced cytokine level" is explained. When referring to the above-described example, the level of inflammatory cytokine in the supernatant of each well is measured by ELISA or such. The cell supernatant of the control is similarly measured. To "contact only with the inducer of cytokine production" means that the inducer of cytokine production, but not the test substance, was contacted with the cells. Although any inflammatory cytokine can be selected as the cytokine to be measured, IFN-γ, TNFα, IL-1, and IL-6 are preferred examples. By comparing the measured values thus obtained with the control and selecting wells in which the measured value of the well supernatant is lower than the control, a substance of interest can be selected from test substances. Since the substance thus selected possibly has a CD61-binding ability and has an inhibitory effect on the production of inflammatory cytokines, it

can be used, as described above, as an inhibitor of inflammatory cytokine production or as a pharmaceutical for preventing or treating inflammatory diseases and hypercytokinemia.

[0037]

After the above-described screening, a secondary screening is further performed and a substance whose activity depends on CD61 can be selected from the substances selected by the aforementioned screening method (hereinafter referred to as a primary selection substance). For example, CD61-expressing cells are seeded into 96-well plates and a primary selection substance and a labeled anti-CD61 antibody are added thereto. The supernatant of each well is used as a sample, a flow cytometry is performed on this sample, and a sample with a lower reaction than the control is selected. Herein, a control is a sample of CD61-expressing cells to which the labeled anti-CD61 antibody has been added, but not the primary selection substance. As a result of the flow cytometry, it can be conceived that, in a sample with a lower reaction than the control, the primary selection substance in the sample competitively inhibits the binding of the anti-CD61 antibody to CD61 on the cell surface. Accordingly, a substance selected by this secondary screening can be said to have a CD61-dependent inhibitory effect on the production of inflammatory cytokines.

[Examples]

[0038]

Herein below, the present invention is more specifically described with reference to Examples, but it is not to be construed as being limited thereto.

[Example 1] Preparation of monoclonal antibodies

The acquisition of functional antibodies that endogenously regulate cytokines was attempted. First, PBMC used as the immunogen were prepared from human blood. Blood from healthy normal subjects were collected into heparin tubes, diluted two fold with RPMI (Sigma R8340), and layered on Histopaque 1077 (Sigma). After centrifugation (800 x G, 30 min), the intermediate layer (leukocyte fraction) was taken out, and erythrocytes were removed by treating with a hemolytic buffer (150 mM NH_4Cl, 1 mM $KHCO_3$, 0.1 mM Na_2EDTA, pH 7.2-7.4) to obtain the PBMCs.

[0039]

Balb/c mice were used as immunized animals. After an injection of 50 μl of Complete Adjuvant (Freund, Iatron) on the day before the initiation of immunization, mice were immunized by injecting the above-described PBMCs in batches of 2.0-5.0 x 10^5 cells each into the foot pad every five days for four times. Mouse foot lymph nodes excised

after the immunization were disrupted to obtain the lymphocytes. These lymphocytes were fused with the P3U1 myeloma cells using polyethylene glycol (PEG, Nakalai).

[0040]

After the hybridomas were cultured in an RPMI (Sigma) selection medium supplemented with 15% FCS and Streptomycin/Penicillin (Gibco BRL) with a final concentration of 5 ng/ml and comprising 2% HAT solution (Gibco BRL), the immunoglobulin-producing ability of the hybridomas was confirmed by a sandwich ELISA, and hybridomas producing immunoglobulins in their supernatant were selected. The sandwich ELISA was performed using an anti-mouse IgG antibody (BS-AU271) sold by MBL as the sensitizing antibody (sensitized at a final concentration of 2 μg/ml) and a peroxidase-labeled anti-mouse IgG antibody (IM-0817) as the detection antibody (diluted 500-fold for detection).

[0041]

For the immunoglobulin-producing hybridomas, culture supernatants which react to PBMCs were selected by flow cytometry. Flow cytometry was performed by staining sample PBMCs (1.0×10^5 cells/sample) with 50 μl of culture supernatant and using an FITC-labeled anti-mouse antibody (available from MBL) as the secondary antibody.

[0042]

Hybridomas producing culture supernatants showing a reactivity toward PBMCs were subjected to the limiting dilution method to obtain monoclonal clones. After purification, the produced monoclonal clone heavy chain (about 55 kDa) and light chain (about 30 kDa) were confirmed by SDS-PAGE to be single bands. From clones confirmed to be monoclonal, a clone whose culture supernatant affects the cytokine production of PBMCs was selected, to obtain the clone producing antibody #33. The method for examining the effect on cytokine production is similar to the method described in Example 2.

[0043]

The monoclonal antibody #33-producing clone was further cultured in RPMI comprising 10% FCS and with a final concentration of 5 ng/ml Streptomycin/Penicillin (Gibco BRL), and culture supernatants for purification were obtained. IgGs in the culture supernatant of the hybridomas (mouse lymphocyte/P3U1) were purified using a Protein A column (Amersham Pharmacia). 50 mM Tris-HCl (pH 8.8), 3M NaCl was used as the washing buffer and 0.17 M Glycine-HCl (pH 2.3) was used as the elution buffer. The eluted IgGs were dialyzed using 50% glycerol/PBS. Antibody #33 was filter-sterilized using a 0.22 μm membrane (Millipore).

[0044]

The antibody isotype was determined using the Isostrip kit (Roche). The isotype of antibody #33 was IgG2a, κ.

[0045]

[Example 2] Analysis of antibody #33's effect on cytokine production by PBMCs

To examine antibody #33's effect on cytokine production by PBMCs, PBMCs were cultured with cytokine production-stimulating substances, antibody #33 was added thereto, and the level of the various cytokines produced by PBMCs was examined.

[0046]

PBMCs isolated from peripheral blood using Histopaque-1077 (Sigma) as described above were used. The isolated cells were seeded into 96-well plates at 1.0×10^5 cells/well. RPMI (Sigma) into which Streptomycin/Penicillin (Gibco BRL) and 10% FCS (Equitech) have been added was used as the medium.

[0047]

As cytokine production-stimulating substances, the CpG motif portion of a non-methylated DNA (GAC30: ACC GAT GAC GTC GCC GGT GAC GGC ACC ACG; custom synthesis requested to QIAGEN), LPS (Sigma) which is a bacterial component of Gram-negative bacteria, and Poly(I:C) (Sigma) which is a double-stranded viral RNA analogue were used. Each stimulating substance was added to the above-described cells under the conditions of final concentrations of 10 μM CpG, 100 ng/ml LPS, and 100 ng/ml Poly(I:C) and cells were cultured for two nights at 37°C under 5% CO_2. Antibody #33 was added to the above-described cells at the same time as the cytokine production-stimulating substances. The culture supernatants were recovered, the production level of the various cytokines was measured using commercially available ELISA kits, and the effect of antibody #33 on cytokine production by PBMCs was investigated. IM-1743 (IFN-γ), IM-1121 (TNFα), IM-0755 (IL-1α), IM-1987 (IL-10), and IM-3582 (IL-1β) (all from MBL) were used as measurement kits.

[0048]

Furthermore, the variation over time of the production level of cytokines by PBMCs was also investigated. Isolated PBMCs were cultured in 12-well plates at 1.0×10^6 cells/well, a fixed amount of culture supernatant was sampled 1, 2, 5, 10, 22, 32, 44, 56, 68, and 80 hours after addition of the stimulant, and the production level of the various cytokines was measured. Antibody #33 was added to the PBMCs at a final concentration of 1 μg/ml at the time of addition of stimulant. Furthermore, as another anti-CD61 antibody, a commercially available anti-CD61 antibody (SZ21, BC), and as a control, a

mouse IgG2a (MBL) isotype control were each added to a final concentration of 1 μg/ml.

[0049]

Results are shown in Figs. 1 to 3. Antibody #33, which is one of the monoclonal antibodies obtained this time, significantly inhibited IFN-γ production by PBMCs (Fig. 1D). Although IFN-γ production is usually detected on or after the 10th hour after stimulation, IFN-γ production was barely observed in the presence of antibody #33 even after 40 hours had elapsed after the stimulation (Fig. 1D). Moreover, antibody #33 was found to also inhibit the production of TNFα and IL-1α, which are inflammatory cytokines (Fig. 2). Given this, a time course was also observed for the inhibitory effect on the production of TNFα and IL-1α, and furthermore on the inhibitory effect on the production of IL-6, which is a typical inflammatory cytokine. As for TNFα, addition of the antibody #33 or the anti-CD61 antibody accelerated the initiation time point of cytokine production; however, a value lower than the control was shown for the total production amount. As for IL-1α and IL-6, the production amount was inhibited from the time of addition of antibody #33 or the anti-CD61 antibody, similarly to IFN-γ (Fig. 1A to C). From these results, antibody #33 was found to be able to simultaneously inhibit the production of many types of inflammatory cytokines.

[0050]

On the other hand, IL-10 is usually detected almost simultaneously with IFN-γ production or slightly later (by the 8th to 10th hour after stimulation). However, it was revealed that, when antibody #33 is administered simultaneously with the stimulation, the time point of initiation of production is by about five hours (Fig. 3A). However, as to the final IL-10 amount produced, no significant difference was observed compared to the control (Fig. 3B).

[0051]

[Example 3] Investigation of the involvement of IL-10 in the inhibitory effect on IFN-γ production by antibody #33

It is generally known that IL-10 inhibits the production of IFN-γ (Non-Patent Document 3). Given this, an investigation was carried out on whether IL-10 is involved in the promotion of IFN-γ production by antibody #33.

[0052]

Poly(I:C) was added to PMBCs, then IL-10 was added at the time of PBMC addition (0 hr), three hours after addition (3 hrs), eight hours after addition (8 hrs), and 20 hours after addition (20 hrs), and the IFN-γ production level was measured at 48 hours after stimulation by ELISA. Antibody #33 was used instead of IL-10 and the production

level of IFN-γ was similarly measured. IL-10 purchased from Sigma was used. Results are shown in Fig. 4A and Fig. 5. When IL-10 was added simultaneously to the stimulation of the PBMCs, IFN-γ production by PBMCs was inhibited, similarly to the Example 2 in which antibody #33 was added. However, when IL-10 was added on and after the 8th hour of stimulation, no inhibitory effect on IFN-γ production was observed (Fig. 4A). On the other hand, an inhibition of IFN-γ production was confirmed even with an administration at 20 hours after stimulation of the PBMCs in the case of antibody #33 (Fig. 5D). Furthermore, when the production level of TNFα, IL-1α, IL-1β, and IL-6 was similarly verified, the cytokine production level was similarly inhibited for these cytokines by the addition of antibody #33 some time after induction of cytokine production. When considering use of antibody #33 in sepsis, this result suggests that antibody #33 would function more effectively than exogenous IL-10 administration in sepsis patients who have already developed hypercytokinemia.

[0053]

Furthermore, Poly(I:C), antibody #33 and a IL-10-neutralizing antibody (R&D, MAB217) were simultaneously added to PBMCs and the produced IFN-γ was measured by ELISA 48 hours after stimulation. Antibody #33 was added at 40 ng/ml, while the IL-10-neutralizing antibody was added at 1000 ng/ml, 50 ng/ml, and 2.5 ng/ml. The result is shown in Fig. 4B. The inhibition of IFN-γ production by antibody #33 was suppressed in a concentration-dependent manner.

[0054]

These results suggest that the inhibitory effect on IFN-γ production by antibody #33 depends on IL-10. In other words, it is thought that when antibody #33 is administered to stimulated PBMCs, the production of IL-10 is promoted, and as a result the production of IFN-γ and inflammatory cytokines is inhibited. Furthermore, antibody #33 is different from IL-10 in that the effect of its addition is recognized even when the period from PMBC stimulation is long; therefore, antibody #33 is assumed to suppress IFN-γ production by PBMC through some other pathway as well, in addition to its IL-10 production promoting effect.

[0055]

[Example 4] Identification of the antigen of antibody #33

To identify the antigen of antibody #33, its reactivity to various human cultured cells was examined by flow cytometry. THP-1 activated macrophage-like cells, HPB-ALL, Jurkat, PM-1, Molt-4, KM-3, HL-60, K562, THP-1, U937, 293T, and HUVEC were used as these cells. The THP-1 activated macrophage-like cells were prepared by culturing and

differentiating for two days the THP-1 human monocyte-like cell line in RPMI (Sigma) including Streptomycin/Penicillin (Gibco BRL) and 10% FCS (Equitech) into which phorbol ester (PMA) (Sigma) has been added to a final concentration of 5 ng/ml .

[0056]

Blocking with 1 mg/ml human IgG (Sigma) in FCS was performed for the various human cultured cells ($1.0x10^5$ cells) and cells were reacted in a PBS solution comprising 2% FCS (Equitech) antibody #33 at a final concentration of 1 μg/ml at 4°C for 30 minutes. After washing twice with PBS comprising 2% FCS (Equitech), cells were further reacted with an FITC-labeled anti-mouse IgG secondary antibody (MBL) at 4°C for 30 minutes. Samples were further washed twice with PBS containing 2% FCS (Equitech) and analyzed with a flow cytometer (ALTRA BC).

[0057]

The results are shown in Fig. 6. It was revealed that antibody #33 strongly reacts with the THP-1 macrophage-like cells differentiated by phorbol ester (Fig. 6C). Furthermore, the T cell line Molt-4 and the human umbilical cord endothelial cells HUVEC also showed reactivity toward antibody #33.

[0058]

An immunoprecipitation experiment using antibody #33 was performed with these THP-1 cells to detect and isolate candidate antigen molecules. As a control, an isotype control was used. First, PBS containing 0.5 mM NHS-LC-Biotin (Pierce) was added to the THP-1 cells so that the cell density becomes 1.0×10^7 cells/ml and this was reacted at 4°C for 1 hour to label the cell surface proteins. After the biotinylated cells were thoroughly washed cell surface proteins were solubilized with PBS containing NP-40 (Nacalai Tesque) and a protease inhibitor cocktail (Sigma). The solubilized proteins were mixed for one hour with Protein A (Amersham Pharmacia) onto which Human IgG (Sigma) have been adsorbed and non-specifically reacting proteins were precipitated and removed. Antibodies were added to the supernatants after the precipitation treatment, let to react at 4°C for one hour or more, and proteins bound to the antibodies were precipitated with Protein A sepharose (Amersham Pharmacia). Proteins adsorbed onto Protein A were eluted, an SDS-PAGE was carried out, and the labeled proteins of interest were detected using Avidine-POD (MBL) and ECL (Amersham Pharmacia). Detection by CBB staining was also performed. As a result, two specific bands were detected at around the molecular weight of 100 kDa. The results are shown in Fig. 7A.

[0059]

From the above-described SDS-PAGE gel, the aforementioned two bands were excised and their analyses were outsourced to APRO Life Science Institute Inc. As a result

of the enzymatic digestion (lysyl endopeptidase) of the gel fragments and the LC-MS/MS analysis and Mascot Search thereafter, it was revealed that, of the two bands, the upper band is CD51 (112 kDa) and the lower band is CD61 (84 kDa).

[0060]

A flow cytometry was performed to determine which of CD51 and CD61, listed as candidate antigen molecules for antibody #33, is the antigen for antibody #33. After PBMCs were stained with antibody #33 and a secondary antibody, they were reacted with a PE-labeled CD51 antibody (BC) or a PE-labeled CD61 antibody (SZ21, BC) at 4°C for 30 minutes and double-stained, after which flow cytometry was performed.

[0061]

The results are shown in Fig. 7B. No cell population double-stained with CD51 and antibody #33 was detected, while the staining properties of CD61 (SZ21, Beckman Coulter) and antibody #33 matched. From this result, it was concluded that the antigen for antibody #33 is CD61. Furthermore, as shown in Fig. 7B, antibody #33 and clone SZ21 do not mutually inhibit the staining, therefore, it was revealed that the epitope recognized by antibody #33 and the epitope recognized by the anti-CD61 antibody SZ21 are different.

[0062]

On the other hand, although its epitope is different from that of antibody #33, an activity to remarkably inhibit the IFN-γ production by PBMCs was also observed with the anti-CD61 antibody SZ21 (Fig. 2A). From this result, it can be inferred that, to inhibit IFN-γ production with an anti-CD61 antibody, it is important to initiate a signal to CD61-expressing cells via CD61 (Integrin βIII), regardless of the epitopes.

[0063]

[Example 5] Examination of CD61-expressing cells

CD61-expressing cells were examined. Among PBMCs, CD61 is known to be expressed on monocytes (Non-Patent Document 7). Antibody #33-positive cells and antibody #33-negative cells among the PBMCs were separated and collected using a cell sorter, and the ability to produce the various cytokines was examined. As a result, it was revealed that antibody #33-positive cells produce IL-10 but not IFN-γ. Conversely, it was revealed that antibody #33-negative cells do not produce IL-10 but produce IFN-γ (data not shown). Considering that IFN-γ is mainly produced by Th1 in peripheral blood, a mechanism can be conceived, in which antibody #33 acts on monocytes to promote IL-10 production, the differentiation of naive T-cells (Th0) into Th1 is thereby inhibited, and consequently, the IFN-γ production level is decreased.

[0064]

[Example 6] Effect of antibody #33 on platelet aggregation

Since CD61 is strongly expressed on platelets, the effect of antibody #33 on platelet aggregation was examined. Blood (anticoagulant: sodium citrate) was centrifuged at room temperature at 800 rpm for 15 minutes to obtain PRP (Platelet Rich Plasma) as a supernatant. For the control, blood was centrifuged at room temperature at 2300 rpm for 15 minutes to obtain PPP (Platelet Poor Plasma) as a supernatant. Antibody #33 at final concentrations of 0, 5, 10, 20, 30, and 50 μg/ml or collagen (a coagulation-promoting substance) was added to the above-described PRP and the transmittance was monitored over time (for a few minutes) using a platelet aggregometer (NBS, HEMA TRACER). Since the transmittance increases when platelets aggregate, the degree of aggregation can be known from the obtained transmittance. As a result, no platelet aggregation activity was detected with antibody #33 (Fig. 8A).

[0065]

To examine the effect of antibody #33 on collagen-induced platelet aggregation, the platelet-aggregating substance collagen (final concentration of 2 μg/ml) was added to PRP, antibody #33 was further added, and measurements using a platelet aggregometer were carried out. As a control, a CD61 antibody (T74) which has a platelet aggregation-inhibitory function was added instead of antibody #33 and measurements were similarly carried out. As for collagens inhibitory effect on aggregation, a slight aggregation inhibition was found (Fig. 8B). These results revealed that antibody #33 has no activity to cause platelet aggregation, but rather has an activity to inhibit aggregation.

[0066]

[Example 7] Examination of antibody #33's inhibitory effect on IFN-γ production ability in the presence of platelets

As described above, since CD61 strongly reacts with platelets, it was examined whether the inhibitory effect by antibody #33 on the IFN-γ production ability is shown even in the presence of platelets. A stimulating substance (Poly(I:C)), antibody #33, a culture medium containing platelets (PRP) were simultaneously added to PBMCs and the produced IFN-γ was measured. PRP was prepared in the same way as in Example 6 and used after replacing plasma with RPMI containing 10% FCS. Antibody #33 was added to the culture medium at a final concentration of 1 μg/ml. PRP was added to the culture medium such that PBMC:PRP (volume) becomes 1:1, 5:1, and 10:1. IFN-γ production level was measured at 48 hours after the addition and as a result, it was confirmed that

antibody #33 inhibits IFN-γ production even in the presence of platelets (Fig. 9).

[0067]

[Example 8] Examination of the inhibitory effect on inflammatory cytokine production by the $F(ab')_2$ fragments of antibody #33

If antibody #33 is administered as is to the human body, it would bind to platelets, and the secondary effects arising from the antibody Fc portions becoming targets of macrophages cannot be ignored. Given this, $F(ab')_2$ fragments of the antibody were prepared and their inhibitory effect on IFN-γ production was examined (Fig. 11A).

[0068]

Antibody #33 IgGs were purified as in the above-described Example 1, then treated with pepsin-agarose (Sigma) in 0.1 M acetic acid (pH 4.3) at 37°C for 20 hours to obtain $F(ab')_2$ fragments. After pepsin degradation, undigested materials having Fc portions were adsorbed with Protein A (Amersham Pharmacia) and the $F(ab')_2$ fraction was purified. After an SDS-PAGE was carried out under non-reducing conditions with the purified $F(ab')_2$ fragments of antibody #33 using a 7.5% acrylamide gel, a CBB staining was performed, and the molecular weight and the degree of purification were verified. When an SDS-PAGE is performed under non-reducing conditions, pepsin-undigested materials can be detected at around 200 kDa while $F(ab')_2$ fragments can be detected at around 150 kDa. Therefore, regarding the molecular weight and the degree of purification of purified $F(ab')_2$ fragments, it was verified whether purified products can be detected at 150 kDa and whether undigested materials (200 kDa) are mixed. The reactivity of the purified $F(ab')_2$ fragments of antibody #33 was verified by flow cytometry (data not shown).

[0069]

Purified $F(ab')_2$ fragments of antibody #33 were added to PBMCs simultaneous to poly(I:C) addition, and the IFN-γ production level after 48 hours was measured by ELISA. $F(ab')_2$ fragments of antibody #33 were added at a final concentration of 10 μg/ml, 1 μg/ml, or 0.1 μg/ml. An inhibitory activity on IFN-γ production was confirmed also with the $F(ab')_2$ fragments of antibody #33, the results of which are indicated in Fig 11B. These results show that the inhibitory effect on IFN-γ production can indeed be expected even in the case of administration of $F(ab')_2$ fragments of antibody #33 to peripheral blood.

[Brief Description of the Drawings]

[0070]

[Fig. 1] A time course of the inhibitory effect of antibody #33 on the production of inflammatory cytokines is shown. The horizontal axis represents the time after addition of

the antibody and the stimulant (pI:C) while the vertical axis represents the level of each cytokine.

[Fig. 2] The inhibitory effect of antibody #33 on the production of inflammatory cytokines is shown.

[Fig. 3] The IL-10 production-inducing effect of antibody #33 is shown. The horizontal axis represents the time after addition of the antibody and the stimulant (pI:C) while the vertical axis represents the level of IL-10.

[Fig. 4A] The variation over time of the inhibitory effect of IL-10 on IFN-γ production, from the time of induction of cytokine production, is shown. The time from induction of cytokine production to IL-10 addition is shown.

[Fig. 4B] The involvement of IL-10 in the inhibition of IFN-γ production by antibody #33 is shown. Numbers in parentheses represent the concentration of antibody #33 or the concentration of IL-10-neutralizing antibody (ng/ml).

[Fig. 5] The inhibitory effect of antibody #33 on the production of various inflammatory cytokines at 48 hours after induction of cytokine production is shown, when the time from the induction of cytokine production until the addition of antibody #33 was changed. The time from induction of cytokine production to addition of antibody #33 is shown.

[Fig. 6] A) The observation of human monocyte-like cells THP-1 with an optical microscope is shown.

B) The observation of THP-1 differentiated into activated macrophage-like cells with phorbol ester (PMA) is shown. Flattened cells are evidence of cell differentiation.

C) A flow cytometry analysis using THP-1 and differentiated THP-1 (TPH-1 + PMA) is shown. The horizontal axis represents the cell number while the vertical axis represents FITC intensity.

[Fig. 7] A) Electrophoresis of the precipitate obtained when differentiated THP-1 cell surfaces were immunoprecipitated with antibody #33 is shown.

B) Flow cytometry analyses of PBMC using antibody #33 and anti-CD51 antibody or antibody #33 and anti-CD61 antibody is shown.

[Fig. 8] An evaluation of the effect of antibody #33 on platelet aggregation is shown.

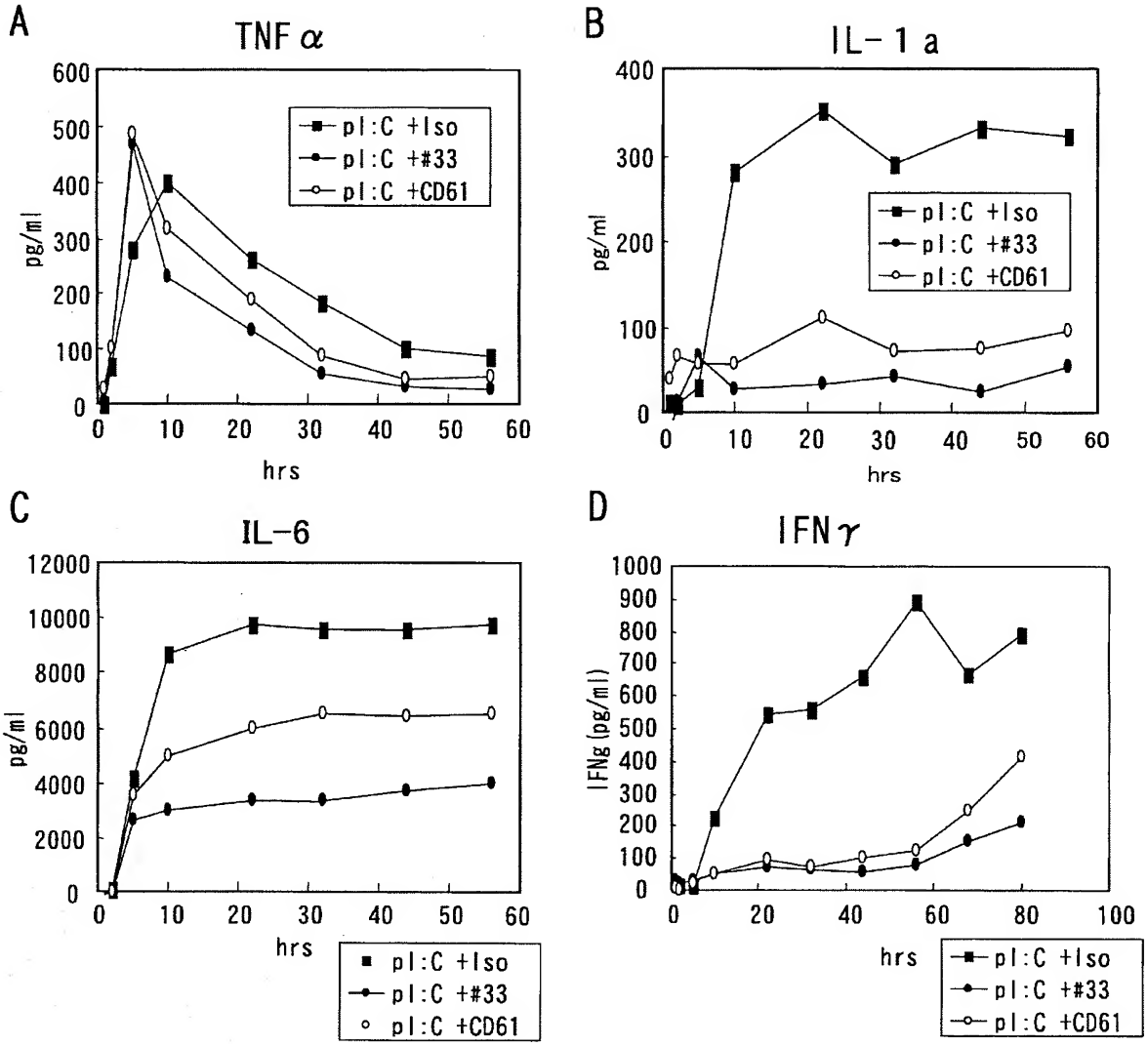
[Fig. 9] The inhibitory effect of antibody #33 on IFN-γ production in the presence of platelets is shown. Numbers in parentheses represent the PRP/PBMC ratio (volume ratio).

[Fig. 10] The inhibitory effect of the $F(ab')_2$ fragment of antibody #33 on the IFN-γ production is shown. Numbers in parentheses represent the antibody #33 concentration

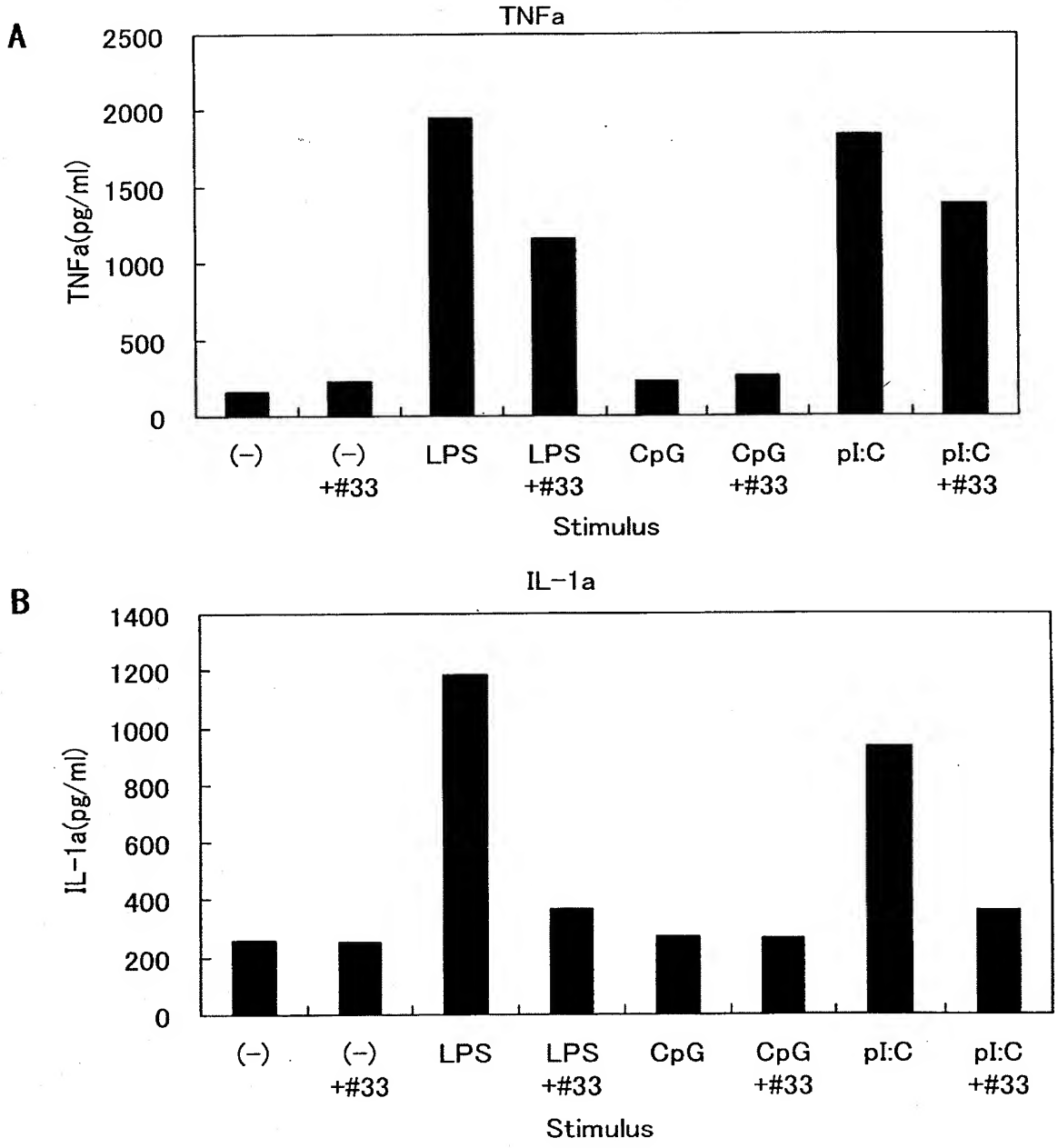
(μg/ml).

[Document Name] Drawings

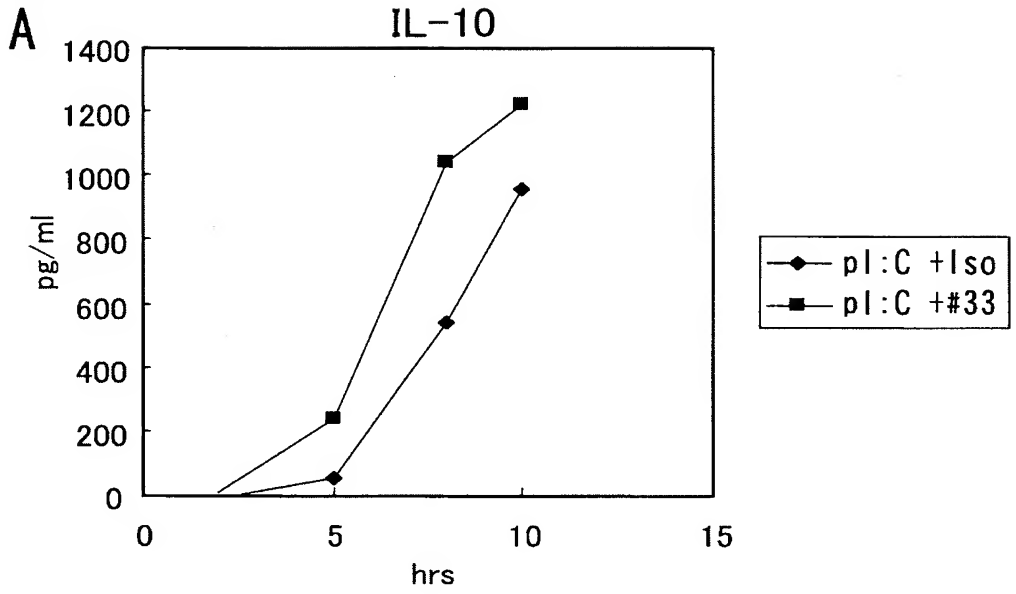
[Fig. 1]

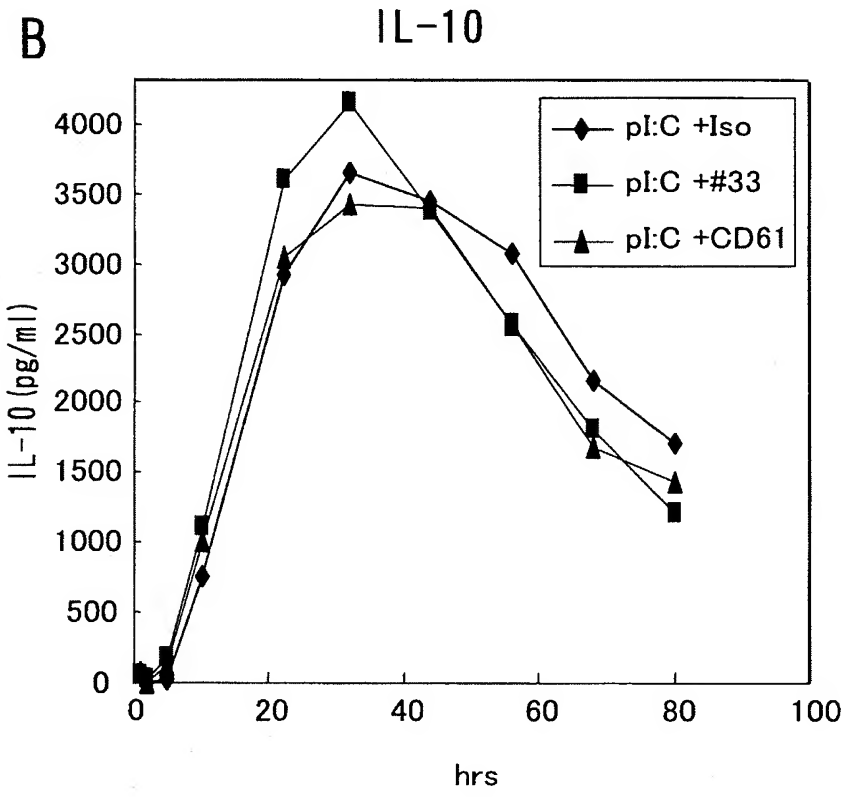


[Fig. 2]

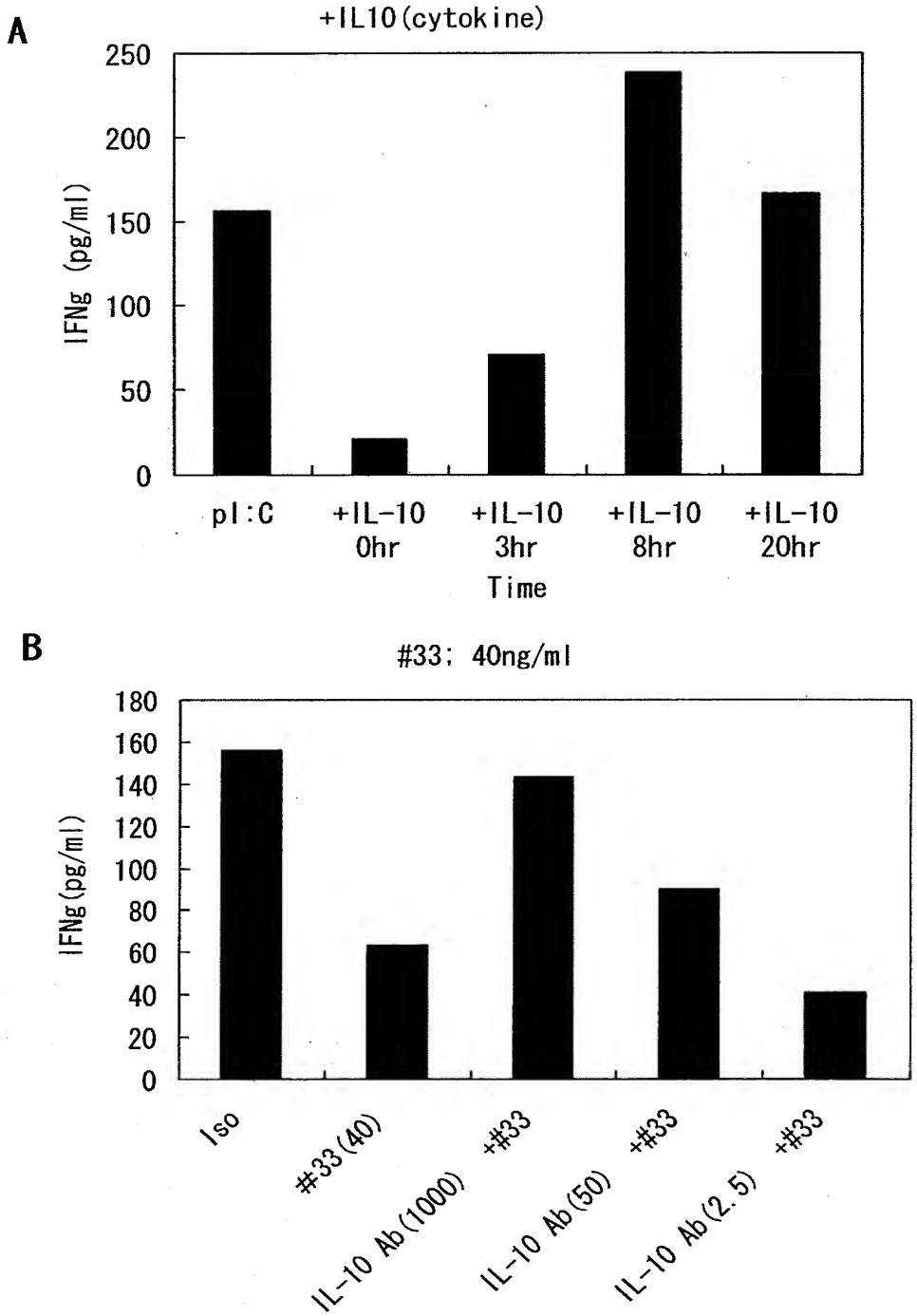

A
TNFa
TNFa(pg/ml)
2500
2000
1500
1000
500
0
(−)
(−) +#33
LPS
LPS +#33
CpG
CpG +#33
pI:C
pI:C +#33
Stimulus
B
IL−1a
IL−1a(pg/ml)
1400
1200
1000
800
600
400
200
0
(−)
(−) +#33
LPS
LPS +#33
CpG
CpG +#33
pI:C
pI:C +#33
Stimulus

[Fig. 3]

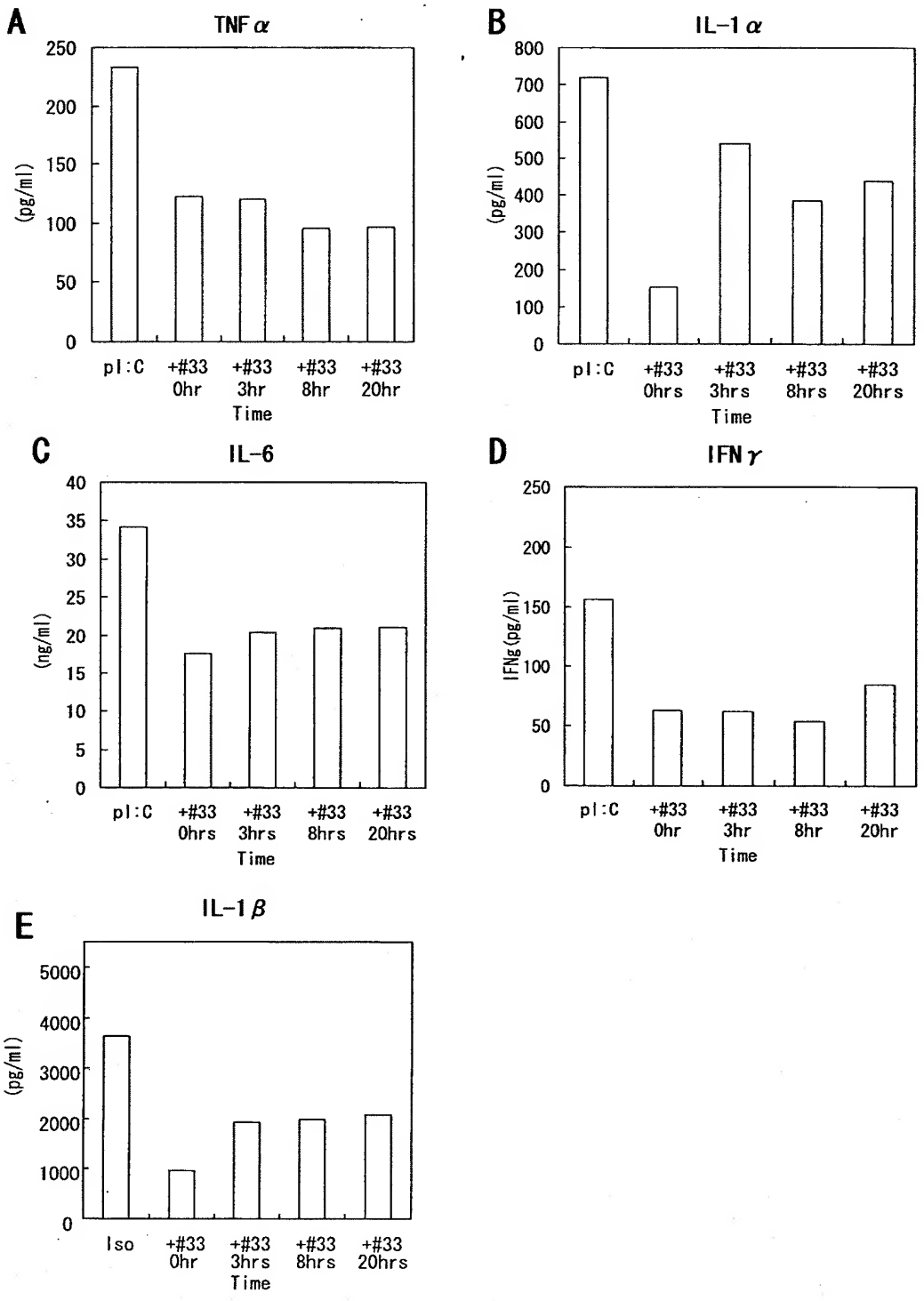

A
IL-10
pg/ml
1400
1200
1000
800
600
400
200
0
0
5
10
15
hrs
pI:C +Iso
pI:C +#33


B
IL-10
IL-10(pg/ml)
4000
3500
3000
2500
2000
1500
1000
500
0
0
20
40
60
80
100
hrs
pI:C +Iso
pI:C +#33
pI:C +CD61

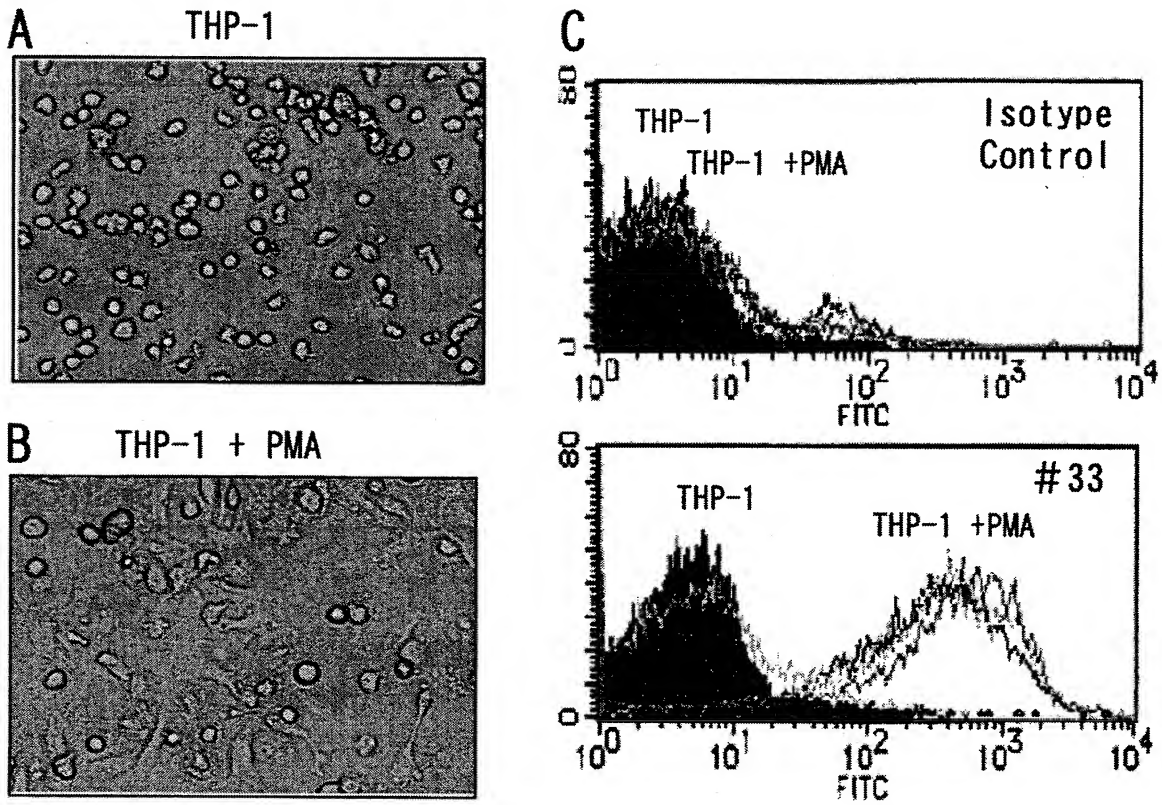
[Fig. 4]


A
+IL10(cytokine)
IFNg (pg/ml)
250
200
150
100
50
0
pI:C
+IL-10 0hr
+IL-10 3hr
+IL-10 8hr
+IL-10 20hr
Time
B
#33; 40ng/ml
IFNg(pg/ml)
180
160
140
120
100
80
60
40
20
0
Iso
#33(40)
+#33 IL-10 Ab(1000)
+#33 IL-10 Ab(50)
+#33 IL-10 Ab(2.5)

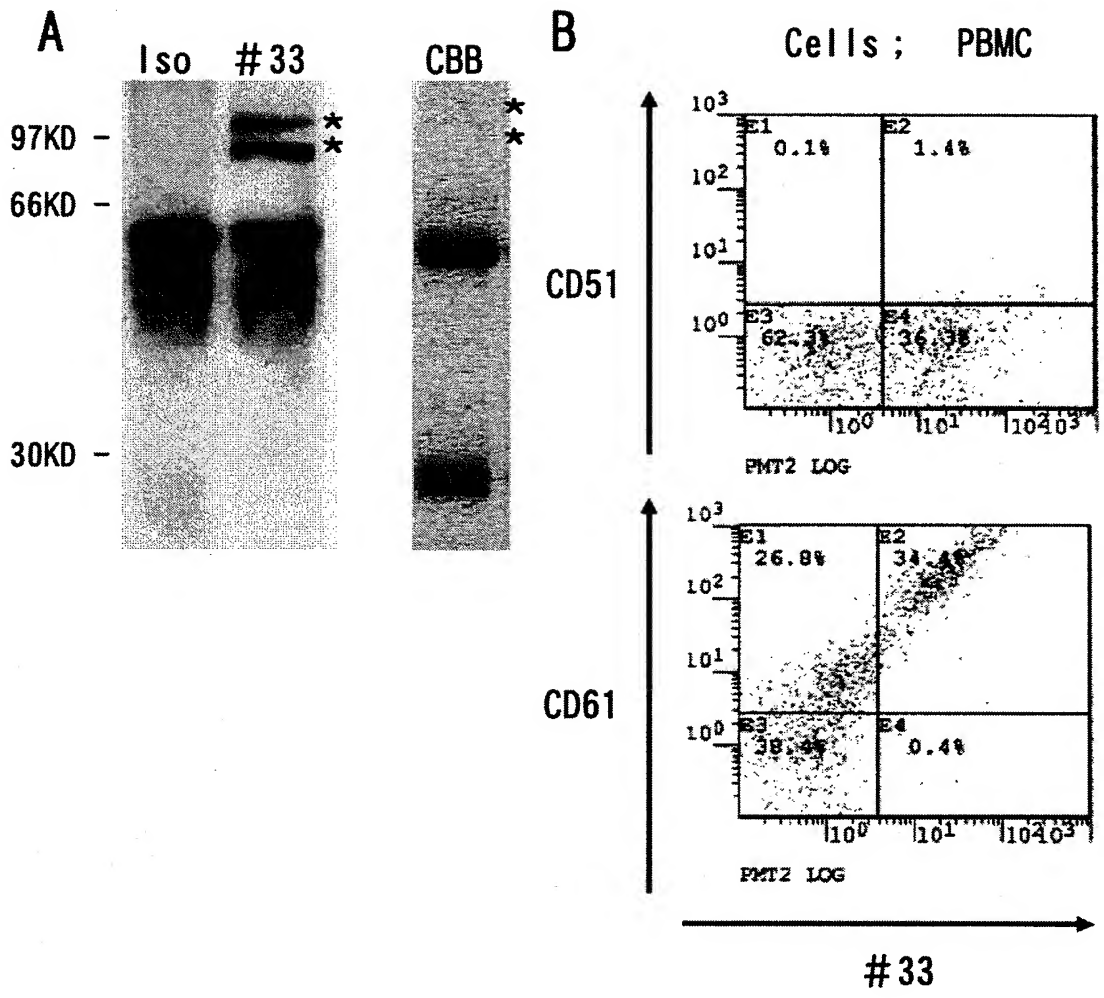
[Fig. 5]


A
TNFα
(pg/ml)
250
200
150
100
50
0
pI:C
+#33 0hr
+#33 3hr
+#33 8hr
+#33 20hr
Time
B
IL-1α
(pg/ml)
800
700
600
500
400
300
200
100
0
pI:C
+#33 0hrs
+#33 3hrs
+#33 8hrs
+#33 20hrs
Time
C
IL-6
(ng/ml)
40
35
30
25
20
15
10
5
0
pI:C
+#33 0hrs
+#33 3hrs
+#33 8hrs
+#33 20hrs
Time
D
IFNγ
IFNg(pg/ml)
250
200
150
100
50
0
pI:C
+#33 0hr
+#33 3hr
+#33 8hr
+#33 20hr
Time
E
IL-1β
(pg/ml)
5000
4000
3000
2000
1000
0
Iso
+#33 0hr
+#33 3hrs
+#33 8hrs
+#33 20hrs
Time

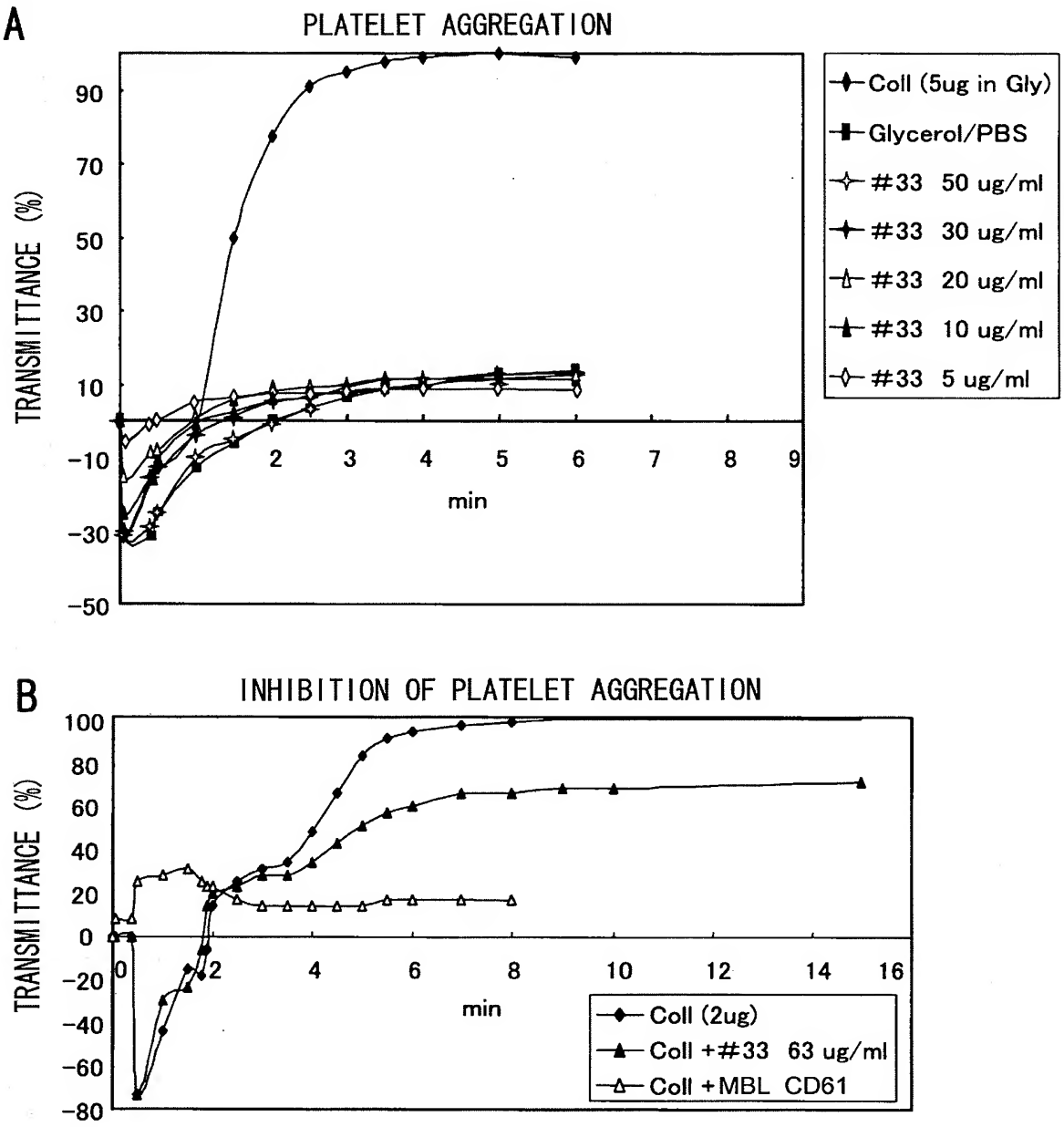
[Fig. 6]


A
THP-1
B
THP-1 + PMA
C
THP-1
THP-1 +PMA
Isotype
Control
80
0
10^0
10^1
10^2
10^3
10^4
FITC
#33

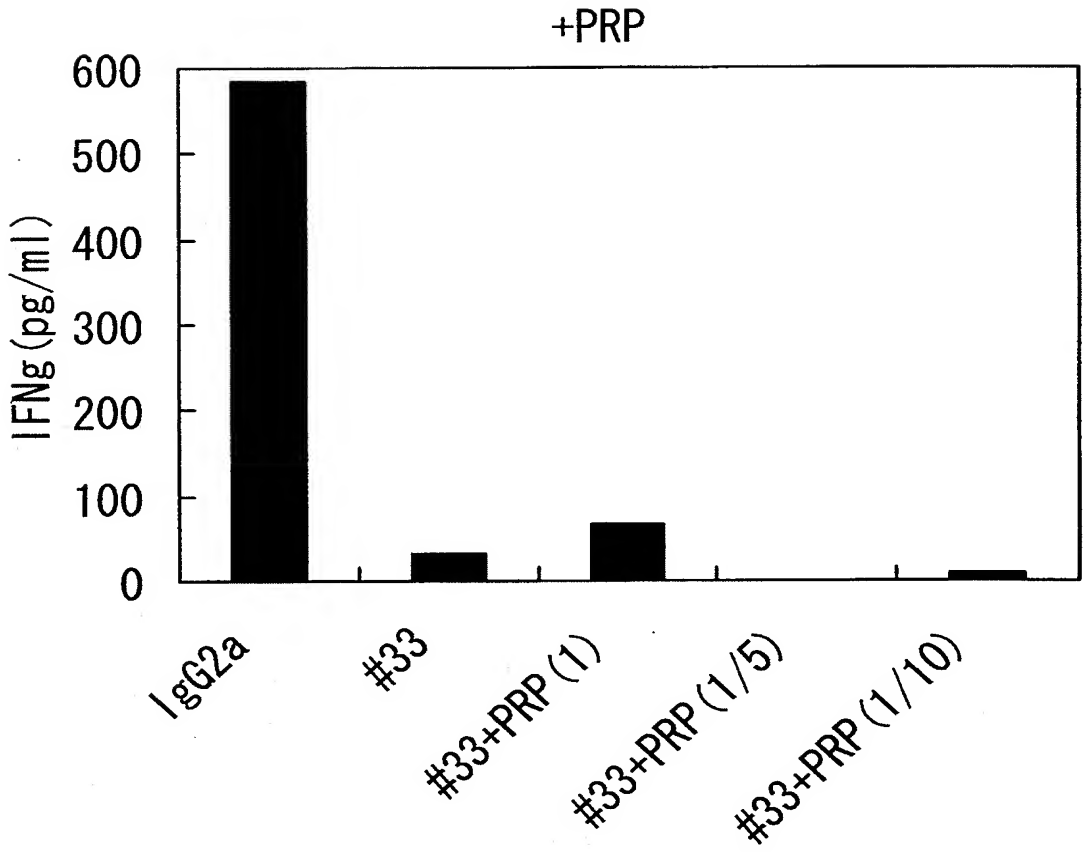
[Fig. 7]


A
Iso
#33
CBB
97KD
66KD
30KD
B
Cells; PBMC
CD51
CD61
#33
E1
0.1%
E2
1.4%
E3
62.3%
E4
36.3%
E1
26.8%
E2
34.4%
E3
38.4%
E4
0.4%
PMT2 LOG
PMT2 LOG

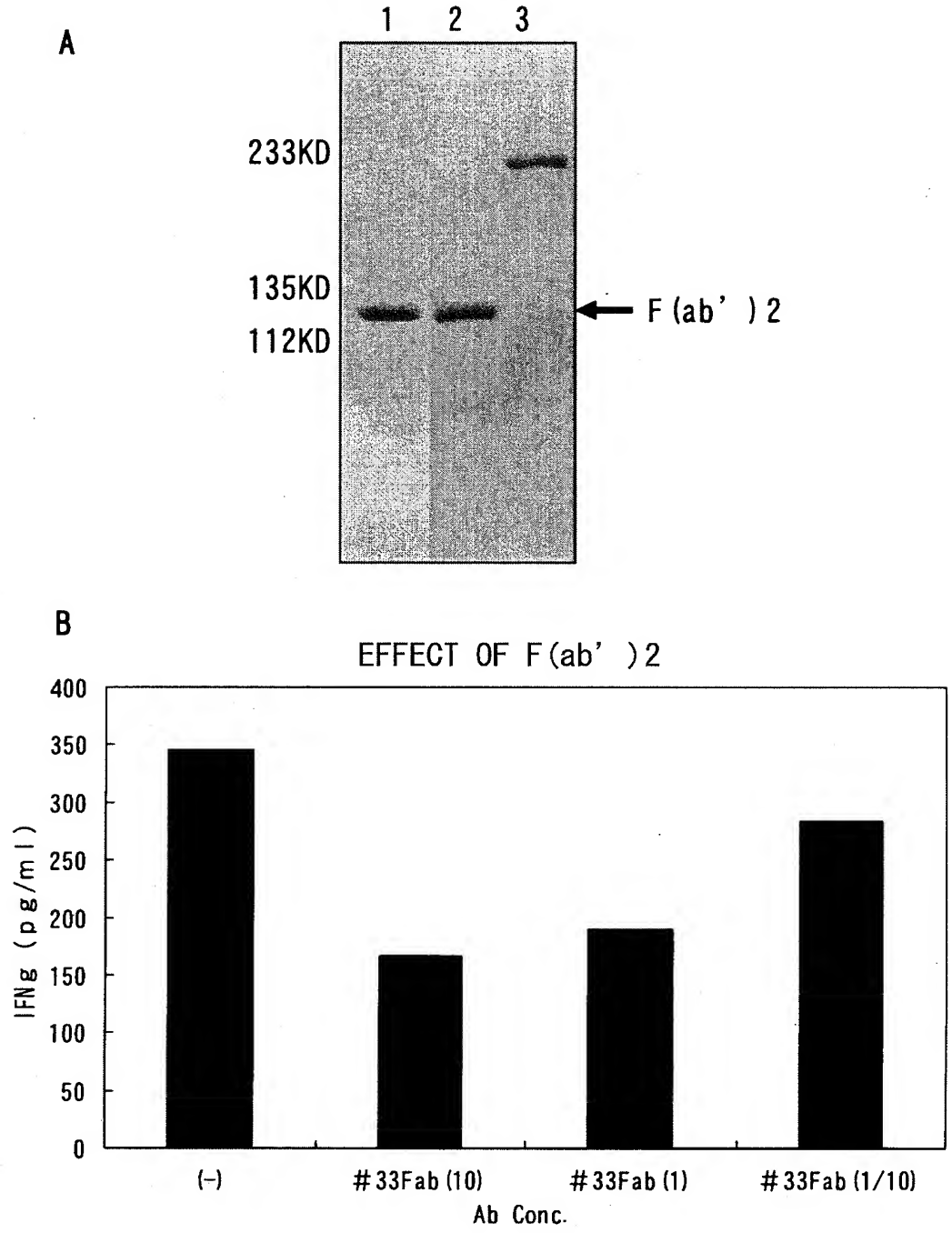
[Fig. 8]


A
PLATELET AGGREGATION
TRANSMITTANCE (%)
90
70
50
30
10
-10
-30
-50
2
3
4
5
6
7
8
9
min
Coll (5ug in Gly)
Glycerol/PBS
#33 50 ug/ml
#33 30 ug/ml
#33 20 ug/ml
#33 10 ug/ml
#33 5 ug/ml
B
INHIBITION OF PLATELET AGGREGATION
TRANSMITTANCE (%)
100
80
60
40
20
0
-20
-40
-60
-80
0
2
4
6
8
10
12
14
16
min
Coll (2ug)
Coll +#33 63 ug/ml
Coll +MBL CD61

[Fig. 9]


+PRP
600
500
400
300
200
100
0
IFNg (pg/ml)
IgG2a
#33
#33+PRP (1)
#33+PRP (1/5)
#33+PRP (1/10)

[Fig. 10]


A
1
2
3
233KD
135KD
112KD
F(ab')2
B
EFFECT OF F(ab')2
IFNg (pg/ml)
400
350
300
250
200
150
100
50
0
(-)
#33Fab(10)
#33Fab(1)
#33Fab(1/10)
Ab Conc.

[Document Name] Abstract

[Abstract]

[Problems to Be Solved] To provide a novel therapeutic drug for inflammatory diseases including sepsis.

[Means for Solving the Problems]

A search was conducted for functional antibodies that endogenously regulate cytokines, focusing on the relationship between inflammatory diseases and cytokines. Mice were immunized with a human peripheral blood monocyte fraction and the obtained antibodies were examined for a cytokine-regulating effect. As a result, of these antibodies, antibody #33 was confirmed to inhibit the production of numerous typical inflammatory cytokines. When, at the same time, the promoting effect of antibody #33 on IL-10 production was examined, it was revealed that antibody #33 has an effect of accelerating IL-10 production but no activity to induce an excessive IL-10 production. The antigen of this promising antibody was verified to be CD61. Therefore, it is conceivable that when anti-CD61 antibodies are applied to the treatment of inflammatory diseases, they would become pharmaceuticals with both a definite efficacy and a high safety.

APPENDIX B

REPORT

Integrin alpha V beta 3 as a target for treatment of rheumatoid arthritis and related rheumatic diseases

R L Wilder

Ann Rheum Dis 2002;**61**(Suppl II):ii96–ii99

A substantial and persuasive body of data now exists that supports the view that integrin alpha V beta 3 plays a critical part in activated macrophage dependent inflammation, osteoclast development, migration, and bone resorption, and inflammatory angiogenesis. All of these processes play an important part in the pathogenesis of rheumatoid arthritis (RA) and related arthropathies. Animal arthritis model data further support these concepts and also suggest that therapeutic antagonism of integrin alpha V beta 3 is worthy of further investigation in RA and related arthropathies. To this end, Vitaxin, also known as MEDI-522, has been developed. Vitaxin is a humanised monoclonal IgG_1 antibody that specifically binds a conformational epitope formed by both the integrin alpha V and beta 3 subunits. It blocks the interaction of alpha V beta 3 with various ligands such as osteopontin and vitronectin. Clinical trials with Vitaxin in patients with RA are in progress.

Although medical treatment for rheumatic diseases such as rheumatoid arthritis (RA) has improved dramatically in recent years (most notably with the development of anti-tumour necrosis factor α agents used in combination with methotrexate), treatment for a substantial number of patients with RA remains suboptimal. Most patients fail to completely respond to these new treatments, and for many other patients, the beneficial effects of these treatments diminish with time. Many of patients with RA still will ultimately require costly joint arthroplasties as an additional effort to relieve pain and to improve or maintain functional abilities. In other words, the cost of the disease to the patients, to the patients' families, and to society remains extremely high. The continued development of new and more effective treatments is clearly warranted.

MEDI-522, also known as Vitaxin, is a new humanised monoclonal IgG_1 antibody that binds human integrin alpha V beta 3 that has now entered clinical development for RA and related diseases. MEDI-522 has a higher affinity for integrin alpha V beta 3 and is more stable than MEDI-523 (also known as Vitaxin 1), which has been phased out of clinical development. MEDI-522 was engineered from the murine monoclonal LM609, an antibody directed against a conformational epitope of the integrin alpha V beta 3 complex. In other words, it specifically recognises an epitope formed by both subunits of integrin alpha V and beta 3 [1] and does not recognise the alpha V or beta 3 subunits alone. In this paper, the biology of integrin alpha V beta 3 and the rationale for its selection as a target for monoclonal antibody treatment of RA will be briefly reviewed.

INTEGRIN ALPHA V BETA 3: WHAT IS IT?

Integrins are heterodimeric transmembrane proteins formed by non-covalent association of alpha and beta subunits. Both subunits are type 1 membrane proteins with large extracellular ectodomains and short cytoplamic tails.[2] In mammals, the integrin family contains at least 18 alpha subunits that associate with at least nine beta subunits. The alpha and beta subunits assemble into at least 24 structurally distinct receptors.[3] Ligands for these integrin receptors are diverse, but all use an acidic residue for recognition by the integrin. Specificity for an individual ligand is determined by additional contacts with the integrin. Ligand binding induces structural changes in the integrin and interactions with other molecules. In general, integrins serve important cellular biosensing roles and convey signals from both outside the cell to inside ("outside-in" signalling) and, vice versa, from inside the cell to outside ('inside-out" signalling).

The integrin alpha V (CD51) subunit can form heterodimers with at least five distinct beta subunits: beta 1, beta 3 (CD61), beta 5, beta 6, and beta 8. The integrin beta 3 subunit, on the other hand, can associate with not only alpha V but also alpha IIb subunits (CD41). This molecular association information is important from the perspective of designing and assessing potential therapeutic monoclonal antibodies. Antibodies that only target alpha V may affect the function of up to five distinct integrins, and antibodies that only target beta 3 may affect both integrins alpha V beta 3 and alpha IIb beta 3. Alpha IIb beta 3 is the major integrin on platelets. As noted above, Vitaxin recognises a specific conformational epitope formed by both the alpha V and beta 3 subunits. In other words, it does not bind integrins alpha V beta 5 or alpha IIb beta 3.

The integrin alpha V beta 3, also known as the vitronectin receptor, consists of a 125 kDa alpha V subunit and a 105 kDa beta 3 subunit. It has been the focus of intensive research because of its major role in several distinct processes, particularly osteoclast mediated bone resorption, angiogenesis and pathological neovascularisation, and tumour metastasis.[4–12] Evidence of the intense interest in this integrin is provided by the recent determination of the crystal structure of the extracellular segment of integrin alpha V beta 3.[13] This work shows that the heterodimeric molecule can exist in either an extended or "flexed" conformation. The available data indicate that the "flexed" or "bent" conformation represents the inactive form.[14] In addition, the crystal structure of alpha V beta 3 binding an Arg-Gly-Asp ligand has also been determined.[15]

INTEGRIN ALPHA V BETA 3: WITH WHAT MOLECULES DOES IT ASSOCIATE?

Integrin alpha V beta 3 has distinct functional properties that are mediated through interactions with a variety of extracellular matrix (ECM) proteins in addition to vitronectin.[6] These ECM proteins include osteopontin, fibronectin, fibrinogen, thrombospondin, proteolysed collagen, von Willebrand factor,

Abbreviations: RA, rheumatoid arthritis; ECM, extracellular matrix

and others. The specific nature of the extracellular interactions of integrin alpha V beta 3 with ECM has additional functional effects on the expression of other cell surface receptors. For example, culturing endothelial cells on vitronectin increases the presence of VEGFR1, as well as VEGFR2, FGFR1, and FGFR2 on the endothelial cell surface. Each of these receptors plays an important part mediating the effects of angiogenic growth factors such as VEGF, FGF1, and FGF2. The increased expression of VEGFR1 and the other receptors is blocked with if anti-alpha V beta 3 and alpha V beta 5 antibodies are added to the culture system. In contrast, plating endothelial cells on fibrin, instead of vitronectin, decreases the expression of these receptors.[16] Thus, these integrins function to transmit signals into the cell, and the cell responds by changing its expression of surface receptors—that is, in this case, the expression of angiogenic growth factor receptors.

Integrin alpha V beta 3 not only modulates the levels of expression of selected cell surface molecules, it also physically associates with a number of important cell surface molecules including VEGFR2, IAP, MTI-MMP, MMP2, and CD47.[17–21] The associations with these various cell surface molecules is modulated by alpha V beta 3 interactions with various ligands, and vice versa, implying that alpha V beta 3 plays a critical part in regulating the localisation or clustering of important cell surface molecules involved in cell adhesion, growth, survival, migration, and invasion through the ECM.

In addition to the ECM and cell surface membrane molecular associations, integrin alpha V beta 3 also interacts with a number of important intracellular signalling molecules. These include paxillin, focal adhesion kinase, caspase 8, and others.[22 23] These interactions also play a part in regulating intracellular signalling, cell migration, cell proliferation, and cell survival.

INTEGRIN ALPHA V BETA 3: WHERE IS IT FOUND? WHAT TISSUES? WHAT CELLS?

Alpha V beta 3 is expressed at low levels in most normal tissues including intestinal, vascular, and smooth muscle cells, but, of particular interest, is that high level expression is limited to bone, the mid-menstrual cycle endometrium, placenta, inflammatory sites, and invasive tumours. The cell types that express high levels of this integrin include mature, bone resorbing osteoclasts and activated macrophages, a small fraction of neutrophils, angiogenic endothelial cells, and migrating smooth muscle cells.[24] These cell types clearly reflect the involvement of integrin alpha V beta 3 in bone resorption, neovascularisation, and inflammation. As pathological osteoclast mediated bone resorption, macrophage dependent inflammation and neovascularisation are characteristic features of diseases such as RA and related arthropathies, these observations provide the first line of supportive evidence that alpha V beta 3 may represent a rationale therapeutic target for these diseases.[25–29] Pathological bone resorption and neovascularisation are also characteristic features of many malignant tumours. Moreover, expression of integrin alpha V beta 3 is also characteristic of several invasive tumour types including melanoma, glioma, ovarian, and breast cancer.[7 30–33] These observations suggest that pharmacological antagonism of the integrin alpha V beta 3 may represent, in addition to rheumatic diseases such as RA, a rational approach to treatment of cancer.

INTEGRIN ALPHA V BETA 3: WHAT DOES IT DO? BIOLOGICAL FUNCTIONS?

From the preceding discussion of the integrin alpha V beta 3, it should be clear that this integrin, through interactions with ECM, various intramembranous and intracellular molecules, modulates the growth, survival, motility, and differentiation of a limited group of cells. These cells include osteoclasts, activated macrophages, and angiogenic endothelial cells. Cellular functional activities are regulated by changes in the affinity and avidity of the integrin, through changes in its phosphorylation state, the nature of ECM ligands, and other factors.[34–36]

The function of the alpha V and beta 3 subunits has also been assessed through studies of gene knockout mice. About 20% of alpha V integrin knockout mice survive to delivery. Placentas from these mice show defects, and the mice have abnormalities in central nervous system and gastrointestinal blood vessels. Cleft palate is also a frequent abnormality. The alpha V integrin subunit associates with beta 1, beta 3, beta 5, beta 6 and beta 8 integrin subunits.[37] Beta 3 integrin knockout mice are fertile and survive.[38] Their important problems are defective platelet aggregation, defective osteoclast resorptive capacity, and osteosclerosis.[39 40] These also have to propensity to permit transplanted tumours to grow more rapidly.[37 41] The report that transplanted tumours grow more rapidly in beta 3 knockout mice has been subject of extensive controversy because the conclusions contrast strikingly with studies with integrin antagonists on tumour growth.[23 42] In other words, conclusions from gene knockout and studies with integrin antagonists on tumour growth have not been consistent without more in depth analysis.

The functional role of integrin alpha V beta 3 has been most extensively studied in the context of osteoclastogenesis and bone resorption, macrophage migration and activation, and angiogenesis. The integrin alpha V beta 3 is highly expressed on activated macrophages and osteoclasts.[43] These cell types are found in abundance at sites of bone destruction in RA patients—that is, activated macrophages are markedly increased in both subchondral bone and inflamed synovial tissues, and osteoclasts are markedly increased in subchondral bone at sites of bone erosion and resorption. In patients with RA, mature osteoclast numbers are highly associated with both periarticular and systemic bone loss.

Osteoclasts are highly specialised and differentiated multinucleated cells related to macrophages. Under normal physiological conditions, mature osteoclasts expressing alpha V beta 3 are continuously generated from immature bone marrow monocytic precursor cells. Stimulatory factors such as macrophage colony stimulating factor (MCSF) and receptor activator of NF-κB (RANK) ligand (RANKL) largely determine this differentiation process. During inflammatory diseases such as RA, tumour necrosis factor α (TNFα) and interleukin 1 (IL1) also significantly amplify osteoclastogenesis and generation of activated macrophages.[43 44] Studies of macrophages and osteoclasts have shown that blocking alpha V beta 3 inhibits adhesion, migration, and, for osteoclasts, bone resorption. Resorbing osteoclasts develop a specialised membrane domain termed the tight sealing zone or clear zone. Alpha V beta 3 and one of its ligands, osteopontin, are enriched in the clear zone of the resorbing osteoclasts. The clear zone is believed to mediate attachment to the bone matrix and the formation of acidic resorption lacunae that are required for bone resorption.[45 46] Other data suggest that alpha V beta 3 is involved in activated macrophage and osteoclast podosome formation. Podosomes are involved in transducing signals that regulate cell attachment, survival, and function internally into the macrophage and osteoclast.[22] Disrupting alpha V beta 3 signalling molecules such as gelsolin impairs podosome formation, cell movements and bone resorption.[47] As osteoclast mediated bone resorption and macrophage dependent inflammation are such a central pathogenic feature of RA, these data provide a strong support for the concept that therapeutic inhibition of alpha V beta 3 is sensible.

Some of highest relative expression levels of alpha V beta 3 are observed on growth factor/cytokine activated endothelial cells, especially blood vessels in granulating wounds or tumours.[7] It is also intensely expressed in inflamed synovial tissues of patients with RA and rabbits with experimental

RA-like arthritis.[48–50] Alpha V beta 3 ligands, such as osteopontin and fibrinogen, are also abundant in rheumatoid synovial tissues. The interaction of these ligands with alpha V beta 3 seems to play an important part in development of the hyperplastic, tumour-like, invasive synovitis that contributes to destruction of bone and cartilage in RA.[51] Endothelial cells in rheumatoid synovium are subject to continuous production of angiogenic stimuli (TNFα, VEGF, FGF1, FGF2), resulting in the expression of alpha V beta 3 on sprouting endothelial cell buds and new blood vessel development.[27] Alpha V beta 3 plays a very important part in this process by facilitating the attachment to and migration through the ECM by the newly developing blood vessels. These observations also support the view that inhibition of alpha V beta 3 in the synovium of RA patients may have therapeutic benefits.

BLOCKADE OF ALPHA V BETA 3 IN PRECLINICAL RA MODELS

A persuasive body of evidence indicates that alpha V beta 3 plays an important part in mediating the migration, differentiation, proliferation, and survival of a limited group of cells that express this integrin. It is particularly noteworthy that the cells that express the highest levels of alpha V beta 3 include activated macrophages, which are involved in producing proinflammatory cytokines, osteoclasts, which mediate inflammatory osteolysis, and endothelial cells, which are involved in pathological neovascularisation. Macrophage dependent processes, inflammatory osteolysis, and neovascularisation are clearly involved in the pathobiology of RA. These observations have stimulated the notion that blocking the function of integrin alpha V beta 3 may have therapeutic benefit in RA, and this hypothesis has been explored to a limited extent in preclinical animal models of inflammatory arthritis.

Intraarticular administration of a cyclic peptide alpha V beta 3 antagonist to rabbits with antigen induced arthritis, a model with features that resemble RA, inhibits synovial angiogenesis, inflammatory cell infiltration, and bone and cartilage destruction. The antagonist treatment also has a disease inhibitory effect when given to animals with chronic disease.[50] In addition, SB273005, a non-peptide antagonist of alpha V beta 3, was reported to significantly reduce symptoms and signs of adjuvant arthritis in the LEW rat. This arthritis model has a very a good record of predicting therapeutic efficacy in patients with RA. SB273005 is also a well documented inhibitor of endothelial cell migration in vitro and a potent inhibitor in several animal models of bone resorption in vivo.[52] Oral dosing with SB273005, started before disease onset or at the time of disease onset, significantly inhibits joint swelling and the destruction of both bone and cartilage (36%–52%, depending on schedule and dose).[53]

More recently, the role of osteopontin has been examined in an experimental RA model. Osteopontin is an extracellular matrix protein containing Arg-Gly-Asp (RGD) sequence, which interacts with alpha V beta 3 integrins. Its expression in the joints of a murine RA model was associated with joint swelling, destruction of the surface structures of the joint based on scanning electron microscopy, and loss of toluidine blue positive proteoglycan content in the articular cartilage in wild type mice. In contrast, osteopontin deficiency prevented the mice from such surface destruction, loss of proteoglycan in the articular joint cartilage, and swelling of the joints. These preclinical results provide further support to the evolving view that alpha V beta 3 is an appropriate target for treatment of RA and related diseases.[54]

Author's affiliations
R L Wilder, Clinical Development, Medimmune, Inc, 35 W Watkins Mill Road, Gaithersburg, MD 20878, USA

Correspondence to: Dr R L Wilder; wilderr@medimmune.com

REFERENCES

1 **Wu H**, Beuerlein G, Nie Y, Smith H, Lee BA, Hensler M, et al. Stepwise in vitro affinity maturation of Vitaxin, an alphav beta3- specific humanized mAb. Proc Natl Acad Sci USA 1998;95:6037–42.
2 **Hynes RO.** Integrins: versatility, modulation, and signaling in cell adhesion. Cell 1992;69:11–25.
3 **Rupp PA**, Little CD. Integrins in vascular development. Circ Res 2001;89:566–72.
4 **Brooks PC**, Clark RA, Cheresh DA. Requirement of vascular integrin alpha v beta 3 for angiogenesis. Science 1994;264:569–71.
5 **Teitelbaum**, SL. Osteoclasts, integrins, and osteoporosis. J Bone Miner Metab 2000;18:344–9.
6 **Horton MA.** The alpha v beta 3 integrin "vitronectin receptor". Int J Biochem Cell Biol 1997;29721–5.
7 **Eliceiri BP**, Cheresh DA. The role of alphav integrins during angiogenesis: insights into potential mechanisms of action and clinical development. J Clin Invest 1999;103:1227–30.
8 **Clover J**, Dodds RA, Gowen M. Integrin subunit expression by human osteoblasts and osteoclasts in situ and in culture. J Cell Sci 1992;103 (Pt 1):267–71.
9 **Helfrich MH**, Nesbitt SA, Horton MA. Integrins on rat osteoclasts: characterization of two monoclonal antibodies (F4 and F11) to rat beta 3. J Bone Miner Res 1992;7:345–51.
10 **Nesbitt S**, Nesbit A, Helfrich M, Horton M. Biochemical characterization of human osteoclast integrins. Osteoclasts express alpha v beta 3, alpha 2 beta 1, and alpha v beta 1 integrins. J Biol Chem 1993;268:16737–45.
11 **Shinar DM**, Schmidt A, Halperin D, Rodan GA, Weinreb M. Expression of alpha v and beta 3 integrin subunits in rat osteoclasts in situ. J Bone Miner Res 1993;8:403–14.
12 **Felding-Habermann B**, O'Toole TE, Smith JW, Fransvea E, Ruggeri ZM, Ginsberg MH, *et al.* Integrin activation controls metastasis in human breast cancer. Proc Natl Acad Sci USA 2001;98:1853–8.
13 **Xiong JP**, Stehle T, Diefenbach B, Zhang R, Dunker R, Scott DL, *et al.* Crystal structure of the extracellular segment of integrin {alpha}V{beta}3. Science 2001;294:339–45.
14 **Beglova N**, Blacklow SC, Takagi J, Springer T. Cysteine-rich module structure reveals a fulcrum for integrin rearrangement upon activation. Nat Struct Biol 2002;9:282–7.
15 **Xiong JP**, Stehle T, Zhang R, Joachimiak A, Frech M, Goodman SL, *et al.* Crystal structure of the extracellular segment of integrin {alpha}V{beta}3 in complex with an arg-gly-asp ligand. Science;2002;296:151–5.
16 **Tsou R**, Isik FF. Integrin activation is required for VEGF and FGF receptor protein presence on human microvascular endothelial cells. Mol Cell Biochem 2001;224:81–9.
17 **Soldi R**, Mitola S, Strasly M, Defilippi P, Tarone G, Bussolino F. Role of alphavbeta3 integrin in the activation of vascular endothelial growth factor receptor-2. EMBO J 1999; 18:882–92.
18 **Weerasinghe D**, McHugh KP, Ross FP, Brown EJ, Gisler RH, Imhof BA. A role for the alphavbeta3 integrin in the transmigration of monocytes. J Cell Biol 1998;142:595–607.
19 **Deryugina EI**, Bourdon MA, Jungwirth K, Smith JW, Strongin AY. Functional activation of integrin alpha V beta 3 in tumor cells expressing membrane-type 1 matrix metalloproteinase. Int J Cancer 2000;86:15–23.
20 **Deryugina EI**, Ratnikov B, Monosov E, Postnova TI, DiScipio R, Smith JW, *et al.* MT1-MMP initiates activation of pro-MMP-2 and integrin alphavbeta3 promotes maturation of MMP-2 in breast carcinoma cells. Exp Cell Res 2001;263:209–23.
21 **Hermann P**, Armant M, Brown E, Rubio M, IshiharaH , Ulrich D, *et al.* The vitronectin receptor and its associated CD47 molecule mediates proinflammatory cytokine synthesis in human monocytes by interaction with soluble CD23. J Cell Biol 1999;144:767–75.
22 **Pfaff M**, Jurdic P. Podosomes in osteoclast-like cells: structural analysis and cooperative roles of paxillin, proline-rich tyrosine kinase 2 (Pyk2) and integrin alphaVbeta3. J Cell Sci 2001;114 (pt 15):2775–86.
23 **Stupack DG**, Puente XS, Boutsaboualoy S, Storgard CM, Cheresh DA. Apoptosis of adherent cells by recruitment of caspase-8 to unligated integrins. J Cell Biol 2001;155:459–70.
24 **Eliceiri BP**, CchereshDA. The role of alphav integrins during angiogenesis. Mol Med 1998;4:741–50.
25 **Jasin HE.** Mechanisms of tissue damage in rheumatoid arthritis In: Koopman WJ, ed. *Arthritis and allied conditions. A textbook of rheumatology.* Philadelphia: Lippincott Williams and Wilkins, 2001:1128–52.
26 **Pap T**, Gay R, Gay S. Mechanisms of joint destruction. In: Firestein GS, Panayi GS, Wollheim FA, eds. *Rheumatoid arthritis. New frontiers in pathogenesis and treatment.* Oxford: Oxford University Press, 2000:189–99.
27 **Koch AE.** The role of angiogenesis in rheumatoid arthritis: recent developments. Ann Rheum Dis 2000;59 (suppl 1):I65-I71.
28 **Goldring SR**, Gravallese EM. Pathogenesis of bone erosions in rheumatoid arthritis. Curr Opin Rheumatol 2000;12:195–9.
29 **Gravallese EM**, Goldring SR. Cellular mechanisms and the role of cytokines in bone erosions in rheumatoid arthritis. Arthritis Rheum 2000;43:2143–51.
30 **Albelda SM**, Mette SA, Elder DE, Stewart R, Damjanovich L, Herlyn M, *et al.* Integrin distribution in malignant melanoma: association of the beta 3 subunit with tumor progression. Cancer Res 1990;50:6757–64.
31 **Brooks PC**, Montgomery AM, Rosenfeld M, Reisfeld RA, Hu T, Klier G, *et al.* Integrin alpha v beta 3 antagonists promote tumor regression by inducing apoptosis of angiogenic blood vessels. Cell 1994;79:1157–64.

32 **Brooks PC**, Stromblad S, Klemke R, Visscher D, Sarkar FH, Cheresh DA. Antiintegrin alpha v beta 3 blocks human breast cancer growth and angiogenesis in human skin. J Clin Invest 1995;96:1815–22.
33 **Carron CP**, Meyer DM, Pegg JA, Engleman VW, Nickols MA, Settle SL, *et al.* A peptidomimetic antagonist of the integrin alpha(v)beta3 inhibits Leydig cell tumor growth and the development of hypercalcemia of malignancy. Cancer Res 1998;58:1930–5.
34 **Boettiger D**, Huber F, Lynch L, Blystone S. Activation of alpha(v)beta3-vitronectin binding is a multistage process in which increases in bond strength are dependent on Y747 and Y759 in the cytoplasmic domain of beta3. Mol Biol Cell 2001;12:1227–37.
35 **Boettiger D**, Lynch L, Blystone S, Huber F. Distinct ligand-binding modes for integrin avb3-mediated adhesion to fibronectin vs vitronectin. J Biol Chem 2001;276:31684–90.
36 **Gottschalk KE**, Gunther R, Kessler H. A three-state mechanism of integrin activation and signal transduction for Integrin alphavbeta3. Chembiochem 2002;3:470–3.
37 **Carmeliet P.** Integrin indecision. Nat Med 2002;8:14–16.
38 **Bouvard D**, Brakebusch C, Gustafsson E, Aszodi A, Bengtsson T, Berna A, *et al.* Functional consequences of integrin gene mutations in mice. Circ Res 2001;89:211–23.
39 **Hodivala-Dilke KM**, McHugh KP, Tsakiris DA, Rayburn H, Crowley D, Ullman-Cullere M, *et al.* Beta3-integrin-deficient mice are a model for Glanzmann thrombasthenia showing placental defects and reduced survival. J Clin Invest 1999;103:229–38.
40 **McHugh**,KP, Hodivala-Dilke K, Zheng MH, Namba N, Lam J, Novack D, *et al.* Mice lacking beta3 integrins are osteosclerotic because of dysfunctional osteoclasts. J Clin Invest 2000;105:433–40.
41 **Reynolds LE**, Wyder L, Lively JC, Taverna D, Robinson SD, Huang X, *et al.* Enhanced pathological angiogenesis in mice lacking beta3 integrin or beta3 and beta5 integrins. Nat Med 2002;8:27–34.
42 **Cheresh DA**, Stupack DG., Integrin-mediated death: an explanation of the integrin-knockout phenotype? Nat Med 2002;8:193–4.
43 **Teitelbaum SL.** Bone resorption by osteoclasts. Science 2000;289:1504–8.
44 **Inoue M**, Ross FP, Erdmann JM, Abu-Amer Y, Wei S, Teitelbaum SL. Tumor necrosis factor alpha regulates alpha(v)beta5 integrin expression by osteoclast precursors in vitro and in vivo. Endocrinology 2000;141:284–90.
45 **Reinholt FP**, Hultenby K, Oldberg A, Heinegard D. Osteopontin—a possible anchor of osteoclasts to bone. Proc Natl Acad Sci USA 1990;87:4473–5.
46 **Nakamura I**, Pilkington MF, Lakkakorpi PT, Lipfert L, Sims SM, Dixon SJ, *et al.* Role of alpha(v)beta(3) integrin in osteoclast migration and formation of the sealing zone. J Cell Sci 1999;112 (pt 22):3985–93.
47 **Chellaiah M**, Kizer N, Silva M, Alvarez U, Kwiatkowski D, Hruska KA. Gelsolin deficiency blocks podosome assembly and produces increased bone mass and strength. J Cell Biol 2000;148:665–78.
48 **Johnson BA**, Haines GK, Harlow LA, Koch AE. Adhesion molecule expression in human synovial tissue. Arthritis Rheum 1993;36:137–46.
49 **Walsh DA**, Wade M, Mapp PI, Blake DR. Focally regulated endothelial proliferation and cell death in human synovium. Am J Pathol 1998;52:691–702.
50 **Storgard CM**, Stupack DG, Jonczyk A, Goodman SL, Fox RI, Cheresh DA. Decreased angiogenesis and arthritic disease in rabbits treated with an alphavbeta3 antagonist. J Clin Invest 1999;103:47–54.
51 **Petrow PK**, Hummel KM, Schedel J, Franz JK, Klein CL, Muller-Ladner U, *et al.* Expression of osteopontin messenger RNA and protein in rheumatoid arthritis: effects of osteopontin on the release of collagenase 1 from articular chondrocytes and synovial fibroblasts. Arthritis Rheum 2000;43:1597–605.
52 **Miller WH**, Keenan RM, Willette RN, Lark MW. Identification and in vivo efficacy of small-molecule antagonists of integrin alphavbeta3 (the vitronectin receptor). Drug Discov Today 2000;5:397–408.
53 **Badger AM**, Blake S, Kapadia R, Sarkar S, Levin J, Swift BA, *et al.* Disease-modifying activity of SB 273005, an orally active, nonpeptide alphavbeta3 (vitronectin receptor) antagonist, in rat adjuvant-induced arthritis. Arthritis Rheum 2001;44:128–37.
54 **Yumoto K**, Ishijima M, Rittling SR, Tsuji K, Tsuchiya Y, Kon S, *et al.* Osteopontin deficiency protects joints against destruction in anti-type II collagen antibody-induced arthritis in mice. Proc Natl Acad Sci USA 2002;99:4556–61.